ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 9

REDACTORES

Y. Reenpää — *Helsinki*
A. Krogh — *Kjöbenhavn*
E. Langfeldt — *Oslo*
G. Liljestrand (editor) — *Stockholm*

COLLABORANTES

G. Ahlgren (Lund), Y. Airila (Helsinki), E. L. Backman (Uppsala), G. Blix (Uppsala), J. Book (Kjöbenhavn), R. Ege (Kjöbenhavn), H. v. Euler (Stockholm), U. S. v. Euler (Stockholm), A. Følling (Oslo), R. Granit (Stockholm), G. Göthlin (Uppsala), E. Hammarsten (Stockholm), E. Hansen (Kjöbenhavn), K. Hansen (Oslo), E. Hohwü-Christensen (Stockholm), G. Kahlson (Lund), F. Leegaard (Oslo), J. Lehmann (Göteborg), J. Lindhard (Kjöbenhavn), E. Lundsgaard (Kjöbenhavn), K. Möller (Kjöbenhavn), R. Nicolaysen (Oslo), S. Örskov (Aarhus), A. V. Sahlstedt (Stockholm), F. Schönheyder (Aarhus), P.E. Simola (Helsinki), T. Teorell (Uppsala), H. Theorell (Stockholm), T. Thunberg (Lund), A. Westerlund (Uppsala), E. Widmark (Lund), A. I. Virtanen (Helsinki)

STOCKHOLM 1945

Reprinted with the permission of Acta Physiologica Scandinavica Karolinska Institutet, Stockholm

JOHNSON REPRINT CORPORATION
111 Fifth Avenue, New York, N.Y. 10003

JOHNSON REPRINT COMPANY LIMITED
Berkeley Square House, London, W.1

First reprinting, 1964, Johnson Reprint Corporation

Printed in the United States of America

VOL. 9. INDEX.

Fasc. 1. (21. II. 1945.)

Pag.

Untersuchungen über Dextran und sein Verhalten bei parenteraler Zufuhr. II. Von ANDERS GRÖNWALL und BJÖRN INGELMAN 1

Influence of Synthetic Polysaccharid Sulfuric Acid Esters on the Thrombocytes in vivo and in vitro. By JØRGEN PIPER 28

Concluding Studies on the Conducting Properties of Human Skin to Alternating Current. By THOMAS ROSENDAL 39

Some Remarks on the Kinetics of the Carotene Loss in Dried Vegetables. By EIGIL ETTRUP PETERSEN and FRITZ SCHØNHEYDER 50

On the Affinity of Pig Pancreas Lipase for Tricaproin in Heterogeneous Solution. By FRITZ SCHØNHEYDER and KIRSTEN VOLQVARTZ 57

Determination of Blood Serum Citric Acid as Acetone. By PER W. KROG 68

Studies on the Initial Increase in O_2-capacity of the Blood at Low O_2-pressure. By ERLING ASMUSSEN and MARIUS NIELSEN 75

The Analgetic Action of Morphine, Eserine and Prostigmine Studied by a Modified Hardy-Wolff-Goodell Method. By SIGVARD FLODMARK and THURE WRAMNER 88

Fasc. 2. (20. III. 1945.)

Some Effects of Acetylcholine, Eserine and Prostigmine when Injected into the Hypothalamus. By NILS EMMELIN and DORA JACOBSOHN 97

A Search for Vagal Afferent Fibres Responding to an Increase of Pressure in the Central Veins. By BO GERNANDT and YNGVE ZOTTERMAN 112

Investigations on the Toxicity and Distribution in the Organism of the Copper (II) Ion in Respect of its Suitability as an Antidote against Hydrocyanic Acid. By CARL-JOHAN CLEMEDSON and BO NORBERG 118

Protein Metabolism of Tissue Cells in vitro. 2. By TAGE ASTRUP, ALBERT FISCHER and MOGENS VOLKERT 134

Treatment of Saliva Samples used for Calcium Analysis. By BODIL SCHMIDT-NIELSEN 162

Pag.
Microdetermination of Calcium in Saliva and Serum. By BODIL SCHMIDT-NIELSEN 166
The Phosphorylation of Glucose in Liver Extracts. By JINGE KJERULF-JENSEN 178
Protein Metabolism of Tissue Cells in vitro. 3. By TAGE ASTRUP and ALBERT FISCHER 183
A Photoelectric Pulse Counter. By NILS P. V. LUNDGREN and YNGVE ZOTTERMAN 191

Fasc. 3—4. (30. V. 1945.)

The Reabsorption of Glycine and Other Amino Acids in the Kidneys of Man. By HANS H. USSING 193
The Determination of Chloroform in Tissues and Blood. By HANS H. USSING 214
Studies on the Action of the Thiamin Destroying Fish Factor. By GUNNAR ÅGREN 221
Rate of Renewal of the Fish Skeleton. By G. HEVESY 234
The Purification and Properties of Aminopolypeptidase from the Hog's Pyloric Mucosa. I. By GUNNAR ÅGREN 248
The Purification of Aminopolypeptidase from the Hog's Pyloric Mucosa. II. By GUNNAR ÅGREN 255
The Purification of Aminopolypeptidase from the Hog's Pyloric Mucosa. III. By GUNNAR ÅGREN 269
Investigations into the Mechanism of the Liberation of Renin in Ischemic Kidneys. By JENS BING 276
On the Structure and Function of the Mammary Glands after Hypophysectomy and Transection of the Hypophyseal Stalk in Rats. By D. JACOBSOHN and A. WESTMAN 284
Further Attempts to Isolate a Gastric Secretory Excitant from the Pyloric Mucosa of Pigs. By BÖRJE UVNÄS 296
On the Purification of the Thiamin-Inactivating Fish Factor. By GUNNAR ÅGREN 306
Reflex Motor Discharges in Single Nerve Fibres of the Frog in Strychnine Poisoning and in other Convulsive States. By EEVA JALAVISTO 313
The Potentiating Effect of Neostigmine and Eserine on the Action of Morphine as Measured by the Straub-Herrmann Reaction. By THURE WRAMNER 336
Gleichzeitige Bestimmung von Äther und Kohlendioxyd in der Atmungsluft. Von PAUL LINDE 341
An Apparatus for Automatic Measurement with Improved Accuracy of Weak Radio-Activities by the Geiger-Müller Counter. By K. ZERAHN 346
Cellulose Sulfuric Acids as Anticoagulants. By TAGE ASTRUP and JØRGEN PIPER 351

Pag.
The Influence of Ionic Strength on the Acid Liver Phosphatase. By SØREN LØVSTRUP JENSEN 357
The Effect of Some Drugs on the Chemoceptive Fibre Activity in the Carotid Sinus Nerve. By B. GERNANDT and Y. ZOTTERMAN 362
Adrenaline Apnoea. By B. GERNANDT, G. LILJESTRAND and Y. ZOTTERMAN 367
Ultrafiltration as a Method of Preparing Blood Plasma for Quantitative Estimation of Histamine. By NILS G. EMMELIN 378
The Piperidine Output in Urine During Muscular Work. By U. S. v. EULER 382

Supplementum XXVII. Central and Indirect Vision of the Light-Adapted Eye. By VIGGO CLEMMESEN.
Supplementum XXVIII. Studies on the Histaminolytic Power of Plasma with special Reference to Pregnancy. By AXEL AHLMARK.

INDEX AUCTORUM.

Pag.

ASMUSSEN, E., and M. NIELSEN, Initial Increase in O_2-capacity of the Blood 75
ASTRUP, T., and M. VOLKERT, Protein Metabolism of Tissue Cells. 2. 134
ASTRUP, T., and A. FISCHER, Protein Metabolism of Tissue Cells. 3. 183
ASTRUP, T., and J. PIPER, Cellulose Sulfuric Acids as Anticoagulants 351
BING, J., Liberation of Renin in Ischemic Kidneys 276
CLEMEDSON, C.-J., and B. NORBERG, Toxicity and Distribution of Copper 118
EMMELIN, N., Ultrafiltration of Histamine 378
EMMELIN, N., and D. JACOBSOHN, Effects of Acetylcholine, Eserine and Prostigmine 97
EULER, U. S. v., Piperidine in Urine 382
FISCHER, A., T. ASTRUP, and M. VOLKERT, Protein Metabolism of Tissue Cells. 2. 134
FISCHER, A., and T. ASTRUP, Protein Metabolism of Tissue Cells. 3. 183
FLODMARK, S., and T. WRAMNER, Analgetic Action of Morphine, Eserine and Prostigmine 88
GERNANDT, B., and Y. ZOTTERMAN, The Bainbridge Reflex 112
GERNANDT, B., and Y. ZOTTERMAN, Drugs on Carotid Sinus 362
GERNANDT, B., G. LILJESTRAND, and Y. ZOTTERMAN, Adrenaline Apnoea 367
GRÖNWALL, A., und B. INGELMAN, Dextran bei parenteraler Zufuhr 1
HEVESY, G., Renewal of Fish Skeleton 234
INGELMAN, B., und A. GRÖNWALL, Dextran bei parenteraler Zufuhr 1
JACOBSOHN, D., and N. EMMELIN, Effects of Acetylcholine, Eserine and Prostigmine 97
JACOBSOHN, D., and A. WESTMAN, Structure and Function of Mammary Glands 284
JALAVISTO, E., Reflex Motor Discharges in Single Nerve Fibres .. 313
KJERULF-JENSEN, J., Phosphorylation of Glucose in Liver Extracts 178
KROG, P. W., Determination of Blood Serum Citric Acid 68
LILJESTRAND, G., B. GERNANDT, and Y. ZOTTERMAN, Adrenaline Apnoea 367
LINDE, P., Äther und Kohlendioxyd in der Atmungsluft 341
LUNDGREN, N. P. V., and Y. ZOTTERMAN, Photoelectric Pulse Counter 191

Pag.
Løvstrup-Jensen, S., Acid Liver Phosphatase 357
Nielsen, M., and E. Asmussen, Initial Increase in O_2-capacity of the Blood 75
Norberg, B., and C.-J. Clemedson, Toxicity and Distribution of Copper 118
Petersen, E. E., and F. Schønheyder, Carotene Loss in dried Vegetables 50
Piper, J., Synthetic Polysaccharid Sulfuric Acid Esters 28
Piper, J., and T. Astrup, Cellulose Sulfuric Acids as Anticoagulants 351
Rosendal, T., Conducting Properties of Human Skin 39
Schmidt-Nielsen, B., Saliva Samples for Calcium Analysis 162
Schmidt-Nielsen, B., Calcium in Saliva and Serum 166
Schønheyder, F., and E. E. Petersen, Carotene Loss in dried Vegetables 50
Schønheyder, F., and K. Volqvartz, Affinity of Pig Pancreas Lipase for Tricaproin 57
Ussing, H. H., Reabsorption of Glycine 193
Ussing, H. H., Chloroform in Tissues and Blood 214
Uvnäs, B., Gastric Secretory Excitant 296
Westman, A., and D. Jacobsohn, Structure and Function of Mammary Glands 284
Volkert, M., T. Astrup, and A. Fischer, Protein Metabolism of Tissue Cells. 2. 134
Volqvartz, K., and F. Schønheyder, Affinity of Pig Pancreas Lipase for Tricaproin 57
Wramner, T., and S. Flodmark, Analgetic Action of Morphine, Eserine and Prostigmine 88
Wramner, T., Potentiating Effect of Neostigmine and Eserine ... 336
Zerahn, K., Measurement of Weak Radio-Activities..... 346
Zotterman, Y., and B. Gernandt, The Bainbridge Reflex 112
Zotterman, Y., and N. P. V. Lundgren, Photoelectric Pulse Counter 191
Zotterman, Y., and B. Gernandt, Drugs on Carotid Sinus 362
Zotterman, Y., B. Gernandt, and G. Liljestrand, Adrenaline Apnoea 367
Ågren, G., Thiamin Destroying Fish Factor 221
Ågren, G., Aminopolypeptidase from Pyloric Mucosa. I. 248
Ågren, G., Aminopolypeptidase from Pyloric Mucosa. II. 255
Ågren, G., Aminopolypeptidase from Pyloric Mucosa. III. 269
Ågren, G., Thiamin-Inactivating Factor 306

Aus dem Physikalisch-Chemischen Institut, Upsala, und dem Tierphysiologischen Institut der Landwirtschaftlichen Hochschule, Upsala.

Untersuchungen über Dextran und sein Verhalten bei parenteraler Zufuhr. II.

Von

ANDERS GRÖNWALL und BJÖRN INGELMAN.

Eingereicht am 6. September 1944.

Physiologische Untersuchungen.

Für die Behandlung von Schockzuständen haben wir vollwertige therapeutische Hilfsmittel im Vollblut, Plasma und Serum. Von gewissen Gesichtspunkten ist indessen diese Therapieform mit Übelständen verknüpft, die sich durch die Anwendung von synthetischen Plasmasubstituten vermeiden lassen. Dieses gilt besonders für die umfangreiche und komplizierte Organisation, die für die Blutentnahme, die Gewinnung von Plasma, nötigenfalls Trocknung des Plasmas, Verwahrung u. s. w. erforderlich ist. Besondere Schwierigkeiten kann die hinreichende Anschaffung von Blut oder Blutplasma bereiten. Aus diesen Gründen wurden schon im ersten Weltkrieg von BAYLISS (1916) Infusionslösungen von Gummi arabicum eingeführt. Auch während des jetzigen Weltkrieges steht die Ausarbeitung eines anwendbaren synthetischen Plasmasubstitutes im Vordergrund des Interesses. Verschiedene Substanzen sind vorgeschlagen und geprüft worden, aber die Mehrzahl war man gezwungen zu verwerfen. Einige haben jedoch eine gewisse Anwendung gefunden.

Die Bedingungen, die ein körperfremdes Kolloid zu erfüllen hat, um eine therapeutische Wirkung bei Schockzuständen auszuüben, sind in Kürze folgende.

Bei allen Formen von drohendem oder manifestem Schock — also nicht nur bei Blutungsschock sondern auch bei Verbrennungsschäden und Quetschungsschäden — gilt es in erster Linie das Volumen der zirkulierenden Blutmasse wieder herzustellen. Fak-

toren wie die gesteigerte Kapillarpermeabilität, die reduzierte Sauerstoffspannung im Gewebe und eine abnorme Verteilung der Elektrolyten zwischen den Zellen und der Intrazellularflüssigkeit sind wohl fundamental im eigentlichen Schocksyndrom, doch nicht in erster Linie Gegenstand einer direkten Therapie, weil sich sekundär die normale Verteilung wieder einstellt, wenn das Blutvolumen seine ursprüngliche Grösse erlangt hat. Um dieses zu erreichen, hat man einerseits kristalloide Lösungen verwendet und andererseits solche, die sowohl Kristalloide als auch irgendeinen Kolloid enthalten. Der Effekt der erstgenannten Lösungen kann gleich nach der Infusion markant sein, ist doch schnell vorübergehend, da die Flüssigkeit bei der automatischen Einstellung des Kolloiddruckes zwischen Blut und Gewebe aus den Blutbahnen ins Gewebe filtriert wird.

Die kolloidalen Lösungen haben indessen einen bedeutend günstigeren Effekt, vorausgesetzt, dass die Partikelgrösse der kolloidalen Substanz so beschaffen ist, dass die Moleküle im Gefässsystem zurückgehalten werden und hinreichende hydrophile Eigenschaften besitzen. Eine Lösung mit diesen Eigenschaften hat nämlich die Fähigkeit nicht nur das fehlende Blutvolumen zu ersetzten, sondern kann auch dazu beitragen, den Kolloiddruck des Blutes auf dem normalen Niveau von 300—400 mm H_2O zu halten. Die untere Grenze für das Molekulargewicht der kolloidalen Substanz hängt natürlich mit der Permeabilität der Kapillaren intim zusammen. Weder normalerweise noch beim Schock tritt Albuminurie auf. Vermutlich wird also ein Molekül von der Grössenordnung des Serumalbumins durch die Glomerulusmembranen zurückgehalten. Die gesteigerte Permeabilität in den übrigen Gewebskapillaren beim Schock ermöglicht indessen das Austreten von Kolloiden dieser Molekulargrösse aus den Gefässen in das Gewebe. Die Erfahrungen bei der Behandlung des traumatischen Schocks mit Plasma lehren, dass trotzdem eine höhere Partikelgrösse der Kolloide bei der Behandlung dieser Zustände nicht erforderlich ist, vermutlich weil die Kapillarpermeabilität verhältnismässig rasch reduziert wird, sobald das Blutvolumen steigt und die Zirkulation sich dem Normalen nähert. Von diesen Überlegungen ausgehend halten wir eine untere Grenze für das Molekulargewicht eines Plasmasubstitutes von 70 000 bis 100 000 für angemessen, jedoch ist diese Grenze u. a. von der Form, der Ladung und dem spezifischen Volumen abhängig.

Für den hämodynamischen Effekt ist auch die Viskosität der

Lösung von Bedeutung. Die relative Viskosität des Plasmas und Blutes beträgt 2 bezw. 5. Die Viskosität darf nicht über eine gewisse Grenze hinaus gesteigert werden, da sonst ein zu grosser Widerstand in der peripheren Zirkulation und damit eine gesteigerte Arbeit für das Herz dem günstigen Effekt entgegenwirken könnte.

Wenn es gilt so grosse Mengen einer fremden Substanz in das Blut einzuführen, muss man die Forderungen an ihre Reinheit und Ungiftigkeit besonders hoch stellen. Das gilt teils die reine Giftwirkung und teils die spezifischen serologischen Erscheinungen von dem Typ, wie sie von der parenteralen Zufuhr von artfremdem Eiweiss oder anderen Substanzen mit antigenen Eigenschaften bekannt sind.

Schliesslich ist es wünschenswert, dass die Substanz im Stoffwechsel in einer Weise teilnimmt, die so wenig wie möglich störend ist. Das Ideal hierfür ist natürlich das arteigene Eiweiss. Substanzen von anderer chemischer Natur sollen jedenfalls den normalen Umsatz oder den normalen Gaswechsel nicht erschweren. Sie dürfen auch keine zu stark sauren oder basischen Eigenschaften besitzen. Man soll unter allen Umständen anstreben, dass ein Plasmasubstitut von artfremden oder synthetischen Kolloiden nicht länger im Blute zirkuliert oder im Körper zurückgehalten wird als absolut nötig ist.

Die erste und vielleicht meist bekannte Plasmaersatzflüssigkeit ist die von Bayliss vorgeschlagene Lösung von Gummi arabicum in Kochsalz. Bayliss selbst setzte grosse Hoffnungen auf diese Substanz und zeigte, dass sie wohl geeignet war, im Tierversuch einen experimentellen Schock zu heben. Die Gummilösungen fanden auch während des ersten Weltkrieges eine weit verbreitete Anwendung in der Krankenpflege der kämpfenden Heere und später auch in der zivilen Krankenpflege, wenn auch gewisse Übelstände damit verbunden waren. Man fand eine erhöhte Tendenz zu Pseudoagglutination der Blutkörperchen und damit eine starke Steigerung der Senkungsreaktion, eine Beiwirkung, deren Bedeutung noch nicht ganz aufgeklärt ist. Weiterhin stellte sich heraus, dass die Gummilösung, obwohl sie die Blutbahnen ziemlich schnell verlässt, im Körper zurückgehalten wird und noch nach Jahren als Aufspeicherungen in der Leber nachgewiesen werden kann. Aus diesem Grunde hat man die Infusion von Gummilösungen jetzt fast ganz aufgegeben. Die früheren Versuche und Erfahrungen mit diesem Präparat deuten indessen

an, dass das Problem eines artifiziellen Plasmasubstitutes keinesfalls unlösbar ist. In der letzten Zeit hat man eine neue Art von Substanzen als Plasmasubstitut versucht, die sogenannten Hochpolymeren, die vor allem von der Kunstharzchemie bekannt sind. Die ersten Versuche sind mit Polyvinylalkohol ausgeführt worden und von JORNS 1937 beschrieben. Seine Anwendbarkeit ist vom Polymerisationsgrad abhängig, und wegen seiner hohen Viskosität muss er in stark verdünnten Lösungen gegeben werden. Schon 0,5 %-ige Lösungen sind von derselben Viskosität wie das Blutplasma. In JORNS Versuchen wurden 3.5 %-ige Lösungen in 7 %-iger Natriumchloridlösung verwendet. Auch hier waren die ersten Mitteilungen optimistisch, aber allmählich entdeckte man Folgeerscheinungen, weshalb der Polyvinylalkohol als Plasmasubstitut nicht durchschlug. HUEPER (1940) und Mitarbeiter betrieben eine eingehende Untersuchung der Toxizität und der pathologischen Befunde. Sie zeigten u. a., dass die Substanz nicht abgebaut wird, sondern sehr lange im Blut und in den Organen verbleibt und gewisse charakteristische Gewebsveränderungen verursacht.

Der nächste Versuch mit einem neuen Hochpolymeren als Plasmasubstitut wurde von HECHT und WEESE (1943) in einer Mitteilung über ein Präparat, Periston genannt, veröffentlicht. (Frühere Bezeichnungen waren Hämodyn und Kollidon). Das Periston ist ein Polyvinylpyrrolidon. Diese Substanz hat eine starke wasserbindende Fähigkeit. Das Molekulargewicht wird zu 20 000 angegeben. Durch Fraktionierungen hat man das Präparat von den kleineren Molekülen befreit und ist zu einem Produkt von höherem mittleren Molekulargewicht gelangt, das nicht so leicht durch die Gefässmembranen hindurchtritt und einen Kolloiddruck von 400 mm H_2O und eine relative Viskosität von 3.0 für eine 3.5 %-ige Lösung besitzt. Toxische Wirkungen sind im Tierversuch nicht gefunden worden, aber beim Menschen scheint man regelmässig eine Nierenreizung mit Albuminurie beobachten zu können. Die Substanz verlässt angeblich das Blut während eines Monats, wenn man eine Transfusionsdosis von der Grösse einer normalen Bluttransfusion gibt. Inwiefern sie im Organismus abgebaut, ausgeschieden oder in den Organen gespeichert wird ist nicht berichtet. Da uns also das Schicksal des Peristons im Organismus noch nicht bekannt ist, sind wir auf die kommenden Mitteilungen hingewiesen, bevor wir uns über eventuelle schädliche Beiwirkungen äussern können.

Im Tierversuch kann ein experimenteller Schock mit Peristonlösung behoben werden; also ist die rein hämodynamische Wirkung, die von der Wasserbindungsfähigkeit innerhalb der Gefässe abhängt, zufriedenstellend. Toxische Wirkungen sind nicht beobachtet worden. Die Blutgerinnung wird nicht beeinflusst, aber die Senkungsreaktion ist stark erhöht. Klinische Versuche sind von KLEES (1943) beschrieben, woraus hervorgeht, dass Periston das beste von den bisher vorgeschlagenen Plasmasubstituten ist. Auch in diesen Versuchen erwies es volle hämodynamische Wirkung. Es gab keine Beiwirkungen mit Ausnahme einer leichten Albuminurie.

Die Anwendbarkeit des Dextrans als Plasmasubstitut.

Eine Substanz, die vorher als Plasmasubstitut noch nicht geprüft worden ist, ist das neutrale Polysaccharid Dextran, dessen Konstitution und chemische und physikalisch-chemische Eigenschaften in Teil I dieser Arbeit näher beschrieben sind (GRÖNWALL und INGELMAN 1944, INGELMAN und SIEGBAHN, 1944). Die Voraussetzung für die Einführung des Dextrans als Plasmasubstitut ist, dass es im Vergleich mit anderen früher beschriebenen Plasmasubstituten offenbare Vorteile hat. Es muss deshalb in erster Linie eine hämodynamische Wirkung ausüben, die mit derjenigen des Serumeiweisses vollkommen vergleichbar ist. In zweiter Linie muss es in grösstmöglichem Masse von den Übelständen frei sein, die den oben genannten und anderen Plasmasubstituten anhaften, wie Pectin (HARTMAN, SCHELLING, HARKINS und BRUSH, 1941) und Gelatine (GORDON, HOGE, LAWSON, 1942). Die Mehrzahl dieser Substanzen sind körperfremd und von dieser Tatsache kann man im wesentlichen ihre Übelstände herleiten. Wohl ist auch das Dextran eine körperfremde Substanz, doch bedeutend weniger körperfremd als irgendein bisher vorgeschlagenes Plasmasubstitut, indem sie nur aus Glucose aufgebaut ist. Die Tatsache, dass sie eine hochpolymere Verbindung ist, braucht nicht abzuschrecken, da wir im Glykogen ein physiologisches Glucosepolymerisat haben. Besonders wichtig ist die Frage über das Schicksal des Dextrans im Organismus nach intravenöser Injektion. Man hat z. B. das Gummi arabicum und den Polyvinylalkohol verwerfen müssen, weil der Körper nicht imstande ist, diese Produkte abzubauen und zu eliminieren. Wie es sich in dieser Hinsicht mit dem Dextran verhält, ist noch nicht

vollkommen aufgeklärt. In in vitro Versuchen haben wir zeigen können, dass das Dextran, wenn es überhaupt von stärkespaltenden Enzymen angegriffen, nur sehr langsam abgebaut wird. Dieses ist zweifellos ein Vorteil, der in gewissem Grade einen langsameren Abbau des Dextrans im Körper garantiert, so dass es eine Zeit lang im Blute verbleibt. Wird beim Abbau Glucose gebildet, kann dieses dem Körper zugute kommen. Werden möglicherweise grössere Spaltprodukte von einem Molekulargewicht von einigen Zehntausend gebildet, können diese durch die Nieren ausgeschieden werden. Auch wenn wir also augenblicklich die Frage, wie das Dextran im Körper abgebaut wird noch nicht beantworten können, wagen wir doch davon auszugehen, dass eine solche Spaltung geschieht. Diese Annahme stützt sich vor allem darauf, dass wir nach sehr starker Überdosierung von Dextran durchaus keine Zeichen für Akkumulation von Dextran in den Organen oder Geweben der Versuchstiere gefunden haben. Später wird näher über diese Versuche berichtet.

Versuche mit anderen Polysacchariden haben ergeben, dass diese ausgesprägte antigene Eigenschaften besitzen können. Wie früher hervorgehoben worden ist (GRÖNWALL und INGELMAN 1944), verursacht das Dextran keine Präzipitinbildung. Man hat deshalb Anlass zu vermuten, dass wiederholte Dextraninjektionen nicht zu anaphylaktischem Schockzustand führen können.

Mehrere Umstände deuten darauf hin, dass sich das Dextran besser als Plasmasubstitut eignen dürfte als früher vorgeschlagene Substanzen. In einer Serie tierexperimenteller Untersuchungen haben wir versucht, diese Frage zu beleuchten.

Versuche mit nicht hydrolysiertem Dextran.

Das Dextran wurde im folgender Weise hergestellt. Ein Substrat aus 15 %-iger Rohzuckerlösung mit etwas Melasse sowie Natriumphosphat versetzt wurde mit Bakterium Leuconostoc mesenteroides geimpft. Nach einer geeigneten Züchtungsdauer wurde das Dextran mit Alkohol ausgefällt. Lösungen davon wurden dialysiert und danach von gröber suspendierten Partikeln befreit, durch Zentrifugieren in einer luftgetriebenen präparativen Zentrifuge bei einer Geschwindigkeit von 22 000 pro Minute. Nach dieser Präparation waren die Lösungen vollkommen farblos und klar. Sie wurden Kaninchen in Konzentrationen zwischen 1 und 3 % und Dosen von 5 bis 40 ml intravenös injiziert. Eine

solche Injektion rief in keinem der Fälle sichere Reaktion bei den Versuchstieren hervor, mit Ausnahme einer mässigen Albuminurie. Wiederholte Injektionen führten allgemeine Verschlechterung mit sich mit Albuminurie, Anurie und schliesslich den Tod. Bei der Sektion wurden an den Nieren sowohl unter der Kapsel als auch im Schnitt stecknadelkopfgrosse, blasse, anämische Herde wahrgenommen. Bei Tieren, die ein Mal gespritzt worden waren, kamen diese Herde spärlich vor. Nach wiederholten Injektionen konnte das ganze Nierenparenchym in dieser Weise verändert sein. Die mikroskopische Untersuchung zeigte Thrombotisierung der Nieren- und Leberkapillaren. Ein Bericht über diesen Befund wird in einer späteren Arbeit erstattet.

Analoge Veränderungen sind nach intravenöser Injektion von Polyvinylalkohol beobachtet worden (HUEPER und Mitarbeiter, 1940). Die Ursache für diese Veränderungen ist früher nicht näher erforscht worden. Wir fanden als wahrscheinliche Ursache eine primäre Thrombotisierung der Glomeruli mit einer umgebenden sekundären anämischen Nekrose. Die Thrombotisierung dürfte entweder durch das Dextran als solches oder auch durch Erythrozytenaggregate, die unter Einwirkung des Dextrans gebildet werden, hervorgerufen sein. Welche Erklärung die richtige ist war nicht zu entscheiden. Möglich erschien uns jedoch die beobachteten Schäden mit einem Dextran von niedrigerem Molekulargewicht vermeiden zu können.

Herstellung von partiell hydrolysiertem Dextran.

Das Dextran kann durch vollständige Hydrolyse zu Glucose abgebaut werden. Bei unvollständiger Hydrolyse kann man Dextran von variierender Molekulargrösse erhalten, wobei das Molekulargewicht immer noch ziemlich hoch sein kann, z. B. von einer Grössenordnung um 200 000. Die im folgenden beschriebenen Präparate wurden durch partielle Hydrolyse mit Salzsäure erhalten. Nach Neutralisation mit Natriumhydroxyd wurde das teilweise abgebaute Dextran mit Alkohol gefällt. Die Fällung wurde in Wasser gelöst und im Cellophansack gegen Wasser dialysiert, wodurch die Produkte geringer Molekulargrösse weggeschaffen wurden. Hiernach wurde die erhaltene Lösung weiterhin durch Filtration durch eine Schicht Kohle und danach mittels Bakterienfiltration gereinigt. Die Präparate wurden auf ihre Reinheit mittels Toxizitätsbestimmungen an Versuchstieren und durch Stickstoffanalysen geprüft. Ausserdem gehörte gewöhnlich zur Bereitung eine Autoklavierung der fertigen Lösung, 20 Min. bei 120°. Hierbei tritt weiterhin eine schwache Hydrolyse ein, die indessen den Grad

der Spaltung nur ganz unbedeutend erhöht. Ultrazentrifugierungsversuche und Diffusionsversuche haben gezeigt, dass diese partiellen Hydrolysate mit Rücksicht auf das Molekulargewicht nicht einheitlich sind.

Die Bedeutung des Hydrolysegrades für die Anwendbarkeit des Dextrans als Plasmasubstitut.

Vorbereitende Versuche zeigten dass man an Versuchstieren partiell hydrolysiertes Dextran intravenös einspritzen kann, ohne dass in den Organen histologisch nachweisbare Veränderungen auftreten. Auch wenn die injizierten Mengen bedeutend waren konnten durchaus keine Schäden von der Art, wie sie das unhydrolysierte Dextran verursachte, entdeckt werden. Ein wichtiger Teil in der weiteren Arbeit bestand nun darin, das höchste Molekulargewicht für das Dextran herauszufinden, bei dem es noch eine zufriedenstellende hämodynamische Wirkung hat, ohne mikroskopisch nachweisbare Gewebsschäden zu verursachen. Dieser Teil der Arbeit ist zusammen mit Med. Dr. NILS GELLERSTEDT, Uppsala, ausgeführt worden und soll später veröffentlicht werden. Es soll indessen schon in diesem Stadium hervorgehoben werden, auch wenn man sich bei der Dextranpräparation unterhalb dieser Grenze hält, ist keine Garantie gegeben, dass nicht andere ungewünschte Beiwirkungen auftreten, die durch ein zu hohes Molekulargewicht ausgelöst werden. Das Problem kann auch folgendermassen formuliert werden. Dextranpräparate mit zu hohem Molekulargewicht geben gewisse Schäden, die vermieden werden müssen. Ist dagegen das Molekulargewicht zu gering verliert das Dextran sowohl die schädlichen Beiwirkungen als auch die physikalisch-chemischen Eigenschaften, die es als Plasmasubstitut geeignet machen. Zwischen diesen beiden Extremen liegt ein Molekulargewichtsintervall, der näher untersucht werden muss, um zu Präparaten mit dem möglichst besten hämodynamischen Effekt unter Vermeidung von schädlichen Beiwirkungen zu gelangen. Eine solche Untersuchung fordert Bestimmungen über den Einfluss von mehreren verschiedenen Faktoren und ist zeitraubend. Die hier mitgeteilten Resultate beleuchten deshalb diese Frage nur teilweise. Sie befassen sich einstweilen im wesentlichen mit gewissen Präparaten, die innerhalb eines begrenzten Molekulargewichtsintervalles gewählt sind.

Wie früher hervorgehoben, ist eine richtige Abpassung der Molekulargrösse zur Permeabilität der Kapillaren von grosser Be-

deutung. Versuche mit Dextranhydrolysaten zeigten schon bald, dass ein Material von niedrigerer Molekulargrösse mit Leichtigkeit durch die Glomerulusmembranen in den Harn abfiltriert werden. Da das Dextran sowohl von Beginn als auch nach der Hydrolyse ein nicht einheitliches Molekulargewicht besitzt, kann nicht vermieden werden, dass es grössere Moleküle enthält, die im Blut zurückgehalten werden und kleinere, die in den Harn abfiltriert werden. Es galt deshalb Dextran herzustellen, das so hochmolekular ist, dass es nicht oder nur zu geringem Teil die Nieren passiert, was man durch eine geeignete Wahl der Hydrolysebedingungen erreichnet kann. Es muss indessen genannt werden, dass es eine Möglichkeit gibt, die kleinen Moleküle durch fraktionierte Fällung mit Alkohol zu entfernen und so Präparate mit begrenztem Molekulargewichtsintervall zu erhalten. Wir haben aber bisher von dieser Komplizierung der Herstellungsmethode Abstand genommen. Erweist es sich notwendig soll sie indessen eingeführt werden.

Die Permeabilität der Glomerulusmembranen ist so wohl bekannt, dass man sich im voraus ungefähr eine Auffassung bilden kann, welches mittlere Molekulargewicht die Dextranhydrolysate haben sollen. So passieren Hämocyanin, Kasein, Serumglobin und Serumalbumin unter physiologischen Verhältnissen nicht die Glomerulusmembranen. Dieses ist indessen der Fall für Ovalbumin und andere Eiweisstoffe mit demselben Molekulargewicht. Das Hämoglobin scheint eine Zwischenstellung einzunehmen, indem es wenigstens bei hinreichend hoher Blutkonzentration die Glomerulusmembranen passiert (Bayliss et al. 1933). Für Proteinstoffe scheint es sich mit der Permeabilität so zu verhalten, dass die Substanzen hindurchtreten können, welche den niedrigsten Molekulargewichtsklassen angehören, nach Svedbergs (1927) Theorie für den multiplen Bau der Proteinmoleküle bis herauf zu einem Molekulargewicht von ca. 35 000—40 000. Substanzen, die der Klasse mit einem Molekulargewicht von um ca. 70 000 angehören, und die folgenden Kategorien mit höherem Molekulargewicht werden zurückgehalten. Die Permeabilitetsgrenze scheint ganz nahe der letzteren Klasse zu liegen, da das Hämoglobin mit einem Molekulargewicht von 68 000 passieren kann. Es muss doch hervorgehoben werden, dass das Molekulargewicht natürlich nicht der einzige entscheidende Faktor ist. Eigenschaften wie das spezifische Volumen, die Form des Moleküls, die Ladungsverhältnisse können eine recht bedeutende Rolle spielen.

Die Permeabilität der Glomerulusmembranen für andere Kolloide als Eiweisstoffe ist indessen kaum bekannt. Eine gewisse Richtschnur konnte durch nicht veröffentlichte Untersuchungen (GRÖNWALL, INGELMAN und MOSIMANN) mit den von MOSIMANN und SVEDBERG (1942) beschriebenen, wasserlöslichen Polysacchariden aus Lärchenholz erhalten werden. Die verwendeten Präparate enthielten Moleküle mit einem Molekulargewicht von 100 000 und 16 000. Diese Polysaccharide sind sehr einheitlich betreffs des Molekulargewichts. Sie sind ungeladen und haben eine etwas langgestreckte Form. Das Verhältnis zwischen Länge und Breite für das Polysaccharidmolekül mit einem Gewicht von 100 000 ist zu 15/1 geschätzt. Diese Polysaccharidmoleküle sind also mehr langgestreckt als z. B. das Serumalbumin. Versuche, die an Kaninchen ausgeführt wurden, haben gezeigt, dass diese Lärchenholzpolysaccharide sehr schnell durch die Glomerulusmembranen aus dem Blut in den Harn filtriert werden. Die aus dem Harn isolierten Polysaccharide hatten dieselbe Sedimentationskonstante wie die ursprünglich intravenös eingespritzten. Man kann deshalb annehmen, dass auch Moleküle mit einem Gewicht von 100 000 die Glomerulusmembranen vollständig ungeschädigt und ohne vorherige Hydrolyse durchtreten können. Von früheren Versuchen wissen wir, dass die Dextranmoleküle sehr langgestreckt sind. Um die Nieren nicht zu passieren müssen sie ein Molekulargewicht haben, das 100 000 übersteigt.

Erfahrungsgemäss hat es sich gezeigt, dass es bei der laufenden Arbeit entbehrlich ist, die Sedimentationskonstante als Ausdruck für den Hydrolysegrad anzuwenden. Um in einfacher Weise den Hydrolysegrad schätzen zu können, haben wir uns als Mass der relativen Viskosität bedient. Es konnte festgestellt werden, dass Lösungen, die bei einer Konzentration von 4 % eine relative Viskosität zwischen 3.5 und 7 haben, nicht die beschriebenen Schäden verursachen und nach Dialyse oder Umfällung mit Alkohol eine relativ geringe Menge feindispersen Materials enthielten. Innerhalb dieses »Viskositätsgebietes», das in Abb. 1 zwischen den Kurven II und IV liegt,

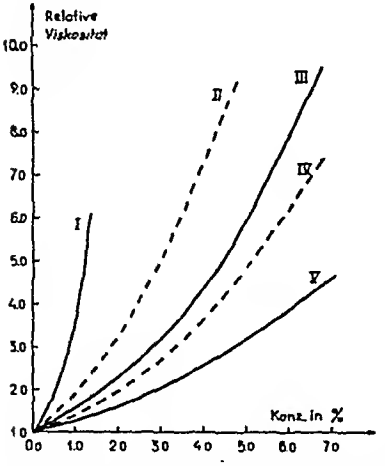


Abb. 1. Die Viskosität verschiedener Dextranpräparate bei variierendem Hydrolysegrad.

hielt sich die Viskosität der meisten verwendeten Präparate. Kurve I ist für eine unhydrolysierte Dextranlösung erhalten worden, während Kurve III einer anwendbaren Infusionslösung entspricht. Eine so weit getriebene Hydrolyse die zu Präparaten mit einer Viskosität entsprechend Kurve V führt, gibt zu kleine Moleküle und soll vermieden werden. Es zeigt sich, dass eine solche Lösung auch nach Dialyse im Cellophansack im wesentlichen so kleine Moleküle enthält, die schnell in den Harn abfiltriert werden.

Methoden zur Bestimmung des Dextrans im Blut und Harn.

Für die Bestimmung des Dextrans im Blut wurden geeignete Modifikationen der Orcinmethode, die polarimetrische Bestimmung, sowie HAGEDORN-JENSENS Titrationsmethode geprüft. Von der erstgenannten sind wir bald zu den beiden letztgenannten Methoden übergegangen. In den hier beschriebenen Versuchen bedienten wir uns der Polarimetermethode in folgender Weise. In einer Blutprobe von 15 ml wurden die Blutkörperchen vom Serum getrennt. Aus diesem wurde das Eiweiss durch Zusatz von Trichloressigsäure gefällt. Nach Dialyse gegen dest. Wasser wurde die Drehung bestimmt. Die Konzentration wurde dann nach Korrektion für die Volumenveränderung und unter Annahme eines Mittelwertes von + 185° für die spezifische Drehung für partiell hydrolysiertes Dextran bestimmt. Im Harn wurde ebenfalls die Dextrankonzentration polarimetrisch nach Dialyse gegen Aq. dest. und nötigenfalls Eindampfen bestimmt. Die HAGEDORN-methode wurde insofern modifiziert, als eine Hydrolyse des Dextrans nach der gewöhnlichen Eiweissfällung mit Zinksulfat und Natriumhydrat eingeschoben wurde. Diese wurde durch 5-stündiges Kochen mit 4 %-iger Schwefelsäure ausgeführt, wonach Neutralisation mit Natronlauge folgte. Eine nähere Beschreibung dieser Methode wird später veröffentlicht.

Dextran im Blut und Harn nach intravenöser Injektion.

Als Tiermaterial kamen Kaninchen und Hunde zur Anwendung. Die Tierversuche gaben im Prinzip übereinstimmende Resultate, weshalb wir uns hier darauf beschränken einen dieser Versuche, einen Hundeversuch mitzuteilen.

Der Hund wog 22.5 kg. Venenpunktion und Aderlass von 100 ml Blut. Danach Infusion von 200 ml 7.3 %-iger Dextranlösung intravenös. Die relative Viskosität der Lösung betrug 10.0. Weder in Verbindung mit der Infusion oder später während der folgenden Tage waren am Tier irgendwelche Schäden noch eine Reaktion gegen das Präparat wahrzunehmen.

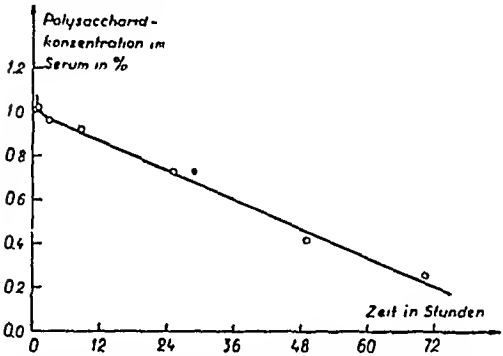


Abb. 2. Dextrankonzentration im Serum eines Hundes nach intravenöser Dextraninjektion.

Das Resultat des Versuches geht aus Abb. 2 hervor, die die Dextrankonzentration im Serum während der Versuchsperiode zeigt. Die Konzentration sinkt sehr schnell unmittelbar nach der Injektion auf ein ziemlich konstantes Niveau, worauf sie während der folgenden Tage langsam absinkt, um nach 4 Tagen den Nullwert zu erreichen. Der erste rasche Abfall ist durch die Ausscheidung des verhältnismässig feindispersen Materials in den Harn erklärt. Die darauf folgende langsame Senkung ist ein Ausdruck für die Eliminierung der grösseren Dextranmoleküle aus den Blutbahnen. In welcher Weise dieses geschieht ist noch nicht vollkommen klargestellt. Im wesentlichen dürfte es sich so verhalten, dass das Dextran auf enzymatischem Wege zu einer gewissen Molekulargrösse abgebaut wird, die das Durchtreten durch die Glomerulusmembranen erlaubt. Das feiner disperse Material wird z. T. durch den Harn ausgeschieden und dürfte zu einem Teil im Körper verbrannt werden. Eine sichere Akkumulation von Dextran in den Organen hat nicht nachgewiesen werden können. Jedenfalls deuten die histologischen Untersuchungen nicht darauf hin. Durch Bestimmungen der Dextrankonzentration im Harn konnte bestätigt werden, dass eine gewisse Menge gleich nach der Injektion ausgeschieden wird und dass während der folgenden Tage Dextran im Harn auftritt. Die Temperatur des Versuchstieres schwankte innerhalb normaler Grenzen. Quantitative Eiweissbestimmungen ergaben keine Albuminurie. Wiederholte Injektionen am selben Tier mit längeren und kürzeren Zeitabständen verursachten auch keine Reaktionen. In keinem Fall konnten Schäden an den inneren Organen beobachtet werden.

Mit Kenntnis der Abbau- und Ausscheidungsverhältnisse des Dextrans ergibt sich die Frage, ob das injizierte Dextran lange genug im Blute verbleibt, um beim Schockzustand die gestörten Zirkulationsverhältnisse effektiv in den Normalzustand zurückzubringen. Mit Ausgangspunkt von diesen Tierversuchen und Literaturangaben lässt sich diese Frage natürlich nicht mit Sicherheit beurteilen. Hier sollen deshalb nur einige allgemeine Überlegungen hinzugefügt werden. Das Dextran kann natürlich das Plasmaeiweiss nicht völlig ersetzten, sondern seine Aufgabe muss es sein vorübergehend einen Ersatz zu bilden und dadurch die Schocksymptome zu beheben. Während dieses geschieht wird das Dextran aus dem Blute entfernt. Es ist deshalb von Bedeutung, dass das Dextran nicht schneller eliminiert wird als der Körper selbst die Zirkulation wieder auf das Normale einzustellen vermag, und dieses muss einerseits durch allgemeine Anpassung des Gefässystems geschehen, andrerseits durch Regeneration des verlorenen Blutvolumens, besonders des Plasmas.

Was die Anpassung des Gefässystems betrifft, so entstehen Schocksymptome gewöhnlich als Folge plötzlicher Veränderungen. Es kann deshalb angenommen werden, dass eine Aufrechterhaltung des Blutvolumens über 12—24 Std. für die Anpassung des Gefässystems vollkommen ausreicht, auch wenn die Fortschaffung des Dextrans aus dem Blut begonnen hat. Die Regeneration des Blutplasmas ist im Tierversuch eingehend untersucht worden, vor allem von WHIPPLE und Mitarbeitern (MADDEN und WHIPPLE, 1940). Es erwies sich, dass das Plasmaeiweiss sehr schnell regeneriert wird, insofern der Organismus nicht durch besondere Verhältnisse an Depoten, stickstoffhaltiger Substanzen ausgearmt ist, die Material für die Synthese von Plasmaproteinen liefern. Auch neuere klinische Untersuchungen sprechen für eine gleichartige rasche Synthese beim Menschen nach Blut und Plasmaverlusten (LANG und SCHWIEGK, 1942, NISSLER, 1942, FELIX, 1940, DUESBERG und SCHWEDER, 1942). Mit Stütze hierauf können wir weder sagen, dass das Dextran aus dem menschlichen Körper mit gleicher Geschwindigkeit eliminiert wird wie im Tierversuch, noch dass die Eliminierungsgeschwindigkeit hinreichend niedrig ist, um dem Plasmaeiweiss Zeit für eine ausreichende Regeneration zu geben, bevor die Dextrankonzentration im Blut zu stark gesunken ist. Wir haben jedoch Gründe, die dafür sprechen, dass es sich wenigstens im Tierversuch so verhält.

Um eine Auffassung über die Grössenordnung derjenigen Moleküle zu bekommen, die durch die Nieren abfiltriert werden, wurde im oben beschriebenen Tierversuch der Harn von der 24. bis 72. Stunde nach Einspritzung gesammelt und das ausgeschiedene Polysaccharid nach Dialyse im Cellophansack durch Fällung mit Alkohol isoliert. Darauf wurde die erhaltene Lösung in der Ultrazentrifuge untersucht. Es zeigte sich hierbei, dass die Substanz betreffs ihres Molekulargewichts nicht einheitlich war, aber dass doch eine Sedimentationskonstante aus den erhaltenen Zentrifugierungsdiagrammen berechnet werden konnte, die nur eine Komponente aufwies. Folgende Werte für die Sedimentationskonstante wurden bei verschiedenen Konzentrationen erhalten:

Tabelle 1.

Konzentration in %	$s \cdot 10^{13}$
1.28	2.6
0.70	2.8
0.35	2.8
0.14	3.4

Eine Extrapolierung der Sedimentationskonstanten auf die Konzentration Null ist in diesem Falle natürlich recht unsicher. Wir schätzen diesen Wert zu $3.6 \cdot 10^{-13}$.

Auch einige Diffusionsversuche wurden an dieser Lösung ausgeführt, wobei die Diffusionskonstante D_A zu $3.8 \cdot 10^{-7}$ bei sowohl 0.70 als auch 0.35 % bestimmt wurde.

(Für die ursprünglich eingespritzte Lösung wurde eine Diffusionskonstante von $2.0 \cdot 10^{-7}$ erhalten. Betreffs der Ausführung der Zentrifugierungen und Diffusionsversuche verweisen wir auf Teil I dieser Arbeit, wo auch D_A definiert ist.)

Setzen wir in SVEDBERGS Formel für die Berechnung des Molekulargewichts die Sedimentationskonstante $= 3.6 \cdot 10^{-13}$, die Diffusionskonstante $= 3.8 \cdot 10^{-7}$ und das partielle spezifische Volumen $= 0.59$ (eine Bestimmung des part. spez. Volumens ist nicht ausgeführt worden, sondern wir rechneten mit dem Wert für das ursprüngliche Dextran) so erhält man ein Molekulargewicht von der Grössenordnung 50 000 bis 60 000. Dieser Wert darf nur als grober Mittelwert aufgefasst werden, da das ausgeschiedene Polysaccharid nicht einheitlich im Molekulargewicht ist. Die ausgeführte Berechnung gibt uns doch eine Auffassung über die Grössenordnung der gespaltenen Dextranmoleküle, die durch

das Nierenfilter ausgeschieden werden können. Der erhaltene Wert liegt wie erwartet zwischen der Permeabilität des Nieren- und Cellophanfilters.

Ein annähernder Wert für das mittlere Molekulargewicht von 50 000—60 000 für das Dextran, welches in den Harn abgeschieden wird, bedeutet selbstverständlich, dass ein Teil der Dextranmoleküle ein wesentlich höheres Molekulargewicht hat. Das höchste Molekulargewicht, das uns ein Mass für die Permeabilität der Glomerulusmembranen liefert, können wir leider nicht angeben. Aber dieses Resultat berechtigt uns doch, ein höheres Molekulargewicht von 100 000—150 000 für die Dextranlösungen zu wählen, die als Plasmasubstitut dienen sollen.

Der Einfluss des Dextrans auf die Senkungsreaktion.

Eine ungewünschte Konsequenz bei Dextraninjektionen ist die Tendenz der roten Blutkörperchen zu Pseudoagglutination (Münzrollenbildung) und damit auch eine Erhöhung der Senkungsreaktion. Aus klinischen Erfahrungen wissen wir, dass eine erhöhte Senkungsreaktion an und für sich nicht schädlich ist, aber bei Dextrankonzentrationen im Blut, wie sie bei Anwendung von Dextranlösungen als Plasmasubstitut in Frage kommen müssen, ist dieser Effekt bedeutend. Wir haben deshalb nicht davon absehen können, dass zur Agglutinationstendenz sekundäre Wirkungen schädlicher Art auftreten können. Ein definitiver Bescheid ist natürlich nicht zu erwarten, bevor die Dextranlösungen in grösseren Mengen am Menschen injiziert sind und die Senkungsreaktion im Verhältnis zum Dextrangehalt des Blutes studiert ist. Als vorbereitende Orientierung haben wir doch in Verbindung mit unseren tierexperimentellen Untersuchungen auch die Senkungsreaktion an Hunden nach Dextraninjektionen bestimmt, sowie in vitro die Einwirkung des Dextrans auf Hunde- und Menschenblut studiert.

Die Resultate der Versuche an Hunden können nicht direkt auf die Verhältnisse am Menschen übergeführt werden. Die Suspensionsstabilität der roten Blutkörperchen ist für die verschiedenen Tierarten sehr verschieden. Diejenige des Menschenblutes ist sehr niedrig und die des Hundeblutes verhältnismässig hoch.

Die Versuche mit Hundeblut sind sowohl in vitro als auch in vivo ausgeführt worden. Die ersteren sind mit Blut von drei verschiedenen Hunden in folgender Weise vorgenommen worden. Venenblut wurde

mit 3.4 %-iger Natriumcitratlösung versetzt, im Verhältnis wie es für die Bestimmung der Senkungsreaktion üblich ist. Danach wurde Dextranlösung und physiologische Kochsalzlösung in variierenden Mengen zugefügt. Die Bereitung der Blutproben geht aus Tabelle 2 hervor, die von einem der Versuche herstammt. Zwei verschiedene Dextranpräparate, Nr. L 30 und L 36 kamen zur Anwendung. Sie enthielten 6 % Dextran in 0.9 % Kochsalz und hatten eine relative Viskosität von 5.0.

Tabelle 2.

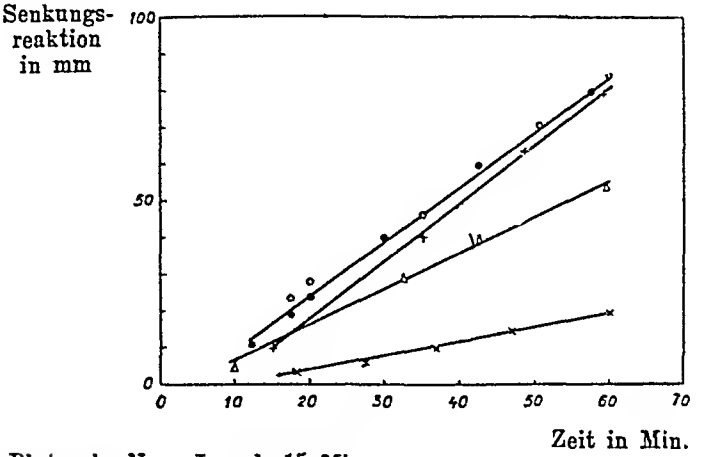
Probe	Hundeblut in ml	3.4%-ige Natrium-citratlg. in ml	6%-ige Dextran-lösung in ml	Physiol. NaCl in ml	Dextran in %
1	1.5	0.5	0.4	—	1
2	1.5	0.5	0.3	0.1	0.75
3	1.5	0.5	0.2	0.2	0.5
4	1.5	0.5	0.1	0.3	0.25
5	1.5	0.5	0.05	0.35	0.125
6	1.5	0.5	0.025	0.38	0.063
7	1.5	0.5	0.013	0.39	0.032
Kontrolle ..	1.5	0.5	—	0.4	—

Mit dem Dextranpräparat L 30 wurden mit Blut von einem Hund die Werte 120 und 30 mm/1 Std. bei einer Dextrankonzentration von 1.0 bezw. 0.5 % Dextran erhalten. Mit L 36 wurden mit Blut von zwei anderen Hunden die Werte 12 und 23 mm/1 Std. mit 1 % Dextran und 11 und 12 mm/1 Std. mit 0.5 % Dextran erhalten. Kontrollbestimmungen der Senkungsreaktion ohne Dextranzusatz gaben für diese drei Tiere die Werte 1.5, 1.5 und 2 mm/1 Std.

In vivo Versuche wurden an einem Hund mit einem Gewicht von 9 kg ausgeführt. Aderlass von 120 ml Blut durch Venenpunktion 12.30 h, 7.1.1944. 13.00 h Injektion von 105 ml 6 %-iger Dextranlösung in 3 %-iger Natriumchloridlösung (Präp. L 30). Blutproben 13.15 h am 7.1., 14.15 h am 7.1., 15.30 h am 7.1., 17.10 h am 8.1., 15.45 h am 9.1., sowie 14.00 h am 11.1. Das Resultat zeigt Abb. 3. In diesem Versuch wurden gleichzeitige Bestimmungen des Dextrangehaltes im Blut nicht vorgenommen. Während der beiden ersten Stunden war die Reaktion unverändert und hielt sich um 35—40 mm/30 Min. und 80—85 mm/1 Std. In den folgenden 2 Tagen wurden niedrigere Werte gemessen, nämlich 54 bezw. 19 mm/1 Std. und nach weiteren zwei Tagen war die Reaktion wieder normal.

In einem anderen Versuch am selben Hund wurde die Senkungsreaktion und die Dextrankonzentration im Blut gleichzeitig be-

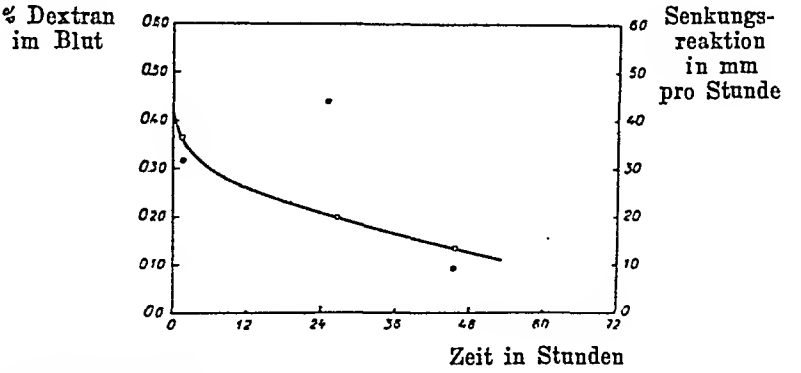
stimmt. Hier kam Präparat 732 b zur Anwendung, Dextrankonzentration 4 % in 3 %-iger Natriumchloridlösung, relative Viskosität 4. Blutproben unmittelbar nach Injektion und nach 2, 25 und 47 Stunden. (Der Dextranwert der ersten Blutprobe ging



● Blutprobe Nr. I nach 15 Min.
\+ » » II » 1 Std. 15 Min.
○ » » III » 2 » 30 »
△ » » IV » 28 » 10 »
× » » V » 50 » 45 »

Abb. 3. Senkungsreaktion beim Hund nach intravenöser Dextraninjektion.

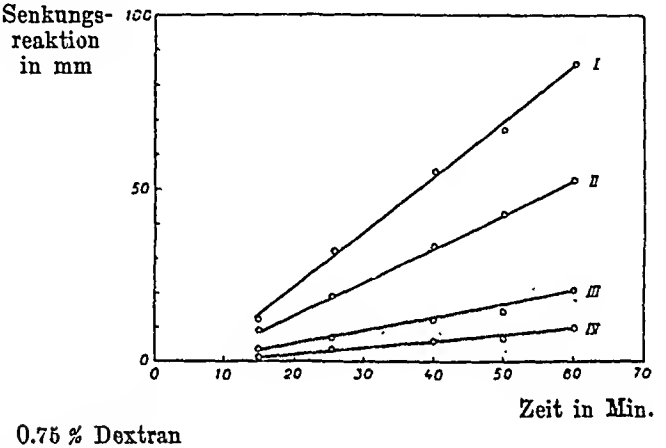
verloren, aber wir wissen aus Erfahrung von anderen ähnlichen Versuchen, dass die Blutkonzentration gleich nach der Injektion wesentlich, ca. 30 %, höher liegt als nach 2 Std.) Das Resultat ist in Abb. 4 zusammengestellt.



● Senkungsreaktion
○ Dextrankonzentration

Abb. 4. Dextrankonzentration im Blut und Senkungsreaktion beim Hund nach intravenöser Dextraninjektion.

Die Versuche mit Hundeblut zeigen in vitro eine recht wesentliche Steigerung der Senkungsreaktion nach Zusatz von Dextran zu Blut. Dasselbe Resultat erhält man in vivo. Hier ist die Erhöhung unmittelbar nach der Dextraninjektion zu registrieren. Sie nimmt während der ersten Stunden nicht ab, wohl aber während der folgenden Tage. Mit der Eliminierung des Dextrans aus dem Blute hat auch die Senkungsreaktion normale Werte erreicht. Eine absolute Parallelität zwischen diesen beiden Werten herrscht indessen nicht. Die Senkungsreaktion fällt nicht parallel mit der Dextrankonzentration während der ersten Stunden nach der In-



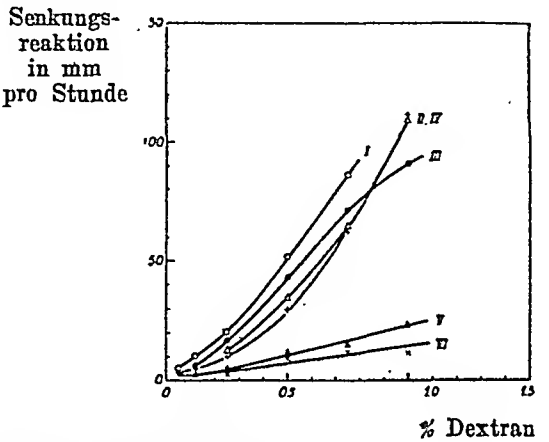
I 0.75 % Dextran
II 0.50 % »
III 0.25 % »
IV 0.12 % »

Abb. 5. Senkungsreaktion in Menschenblut verschiedener Dextrankonzentration.

jektion und im Versuch Abb. 4 ist sie nach 25 Std. höher als unmittelbar nach der Injektion. Die näheren Ursachen hierfür sind noch nicht ermittelt.

Die Versuche am Menschenblut umfassten Bestimmungen der Senkungsreaktion bei variierendem Dextranzusatz und Untersuchungen der Pseudoagglutination bei verschiedener Dextran- und Elektrolytenkonzentration. Bei der Bestimmung der Senkungsreaktion wurde in der Weise verfahren wie schon für die Versuche mit Hundeblut beschrieben. Blut von drei verschiedenen Versuchspersonen kam zur Untersuchung, wobei die oben genannten Präparate L. 30 und L. 36 zur Anwendung kamen, das erstere in einem, das letztere in zwei Fällen. Das Resultat solcher Versuche wird in Abb. 5 wiedergegeben. Die Senkungsreaktion mit Präp. L. 30 beträgt nach einer Stunde bei einer

Dextrankonzentration von 0.75 % 85 mm. 0.5 % Dextran gibt nur den Wert 52 mm. Sowohl mit diesem Präparat als auch mit L. 36 wurde für Menschenblut eine stärkere Beeinflussung der Senkungsreaktion beobachtet als für Hundeblut. Dieses veranschaulicht Abb. 6, wo die Werte für die Senkungsreaktion aller sechs Versuche nach einer Stunde der Dextrankonzentration gegenübergestellt sind.

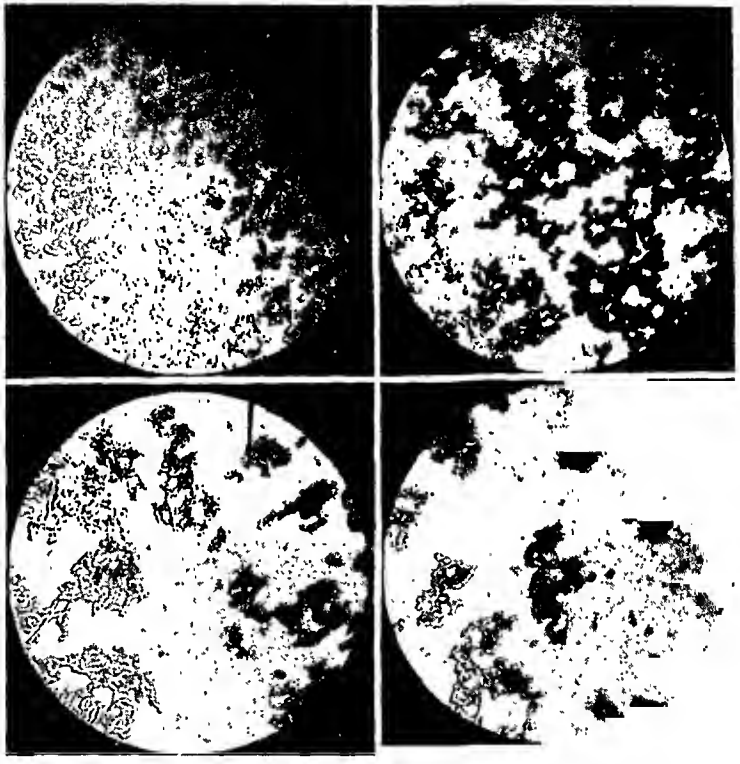


○	I	Menschenblut.	Prep.	L 30
●	III	»	»	L 36
△	IV	»	»	L 36
+	II	Hundeblut.	»	L 30
▲	V	»	»	L 36
×	VI	»	»	L 36

Abb. 6. Senkungsreaktion in Menschen- und Hundeblut verschiedener Dextrankonzentration.

Die in den Blutproben mit Dextranzusatz beobachtete Pseudoagglutination kann recht stark sein und scheinbar den Eindruck wirklicher Agglutination geben. Der Effekt ist augenfälliger je höher die Dextrankonzentration ist. Er ist jedoch auch von der Grösse der Dextranmoleküle abhängig und sinkt mit sinkendem Molekulargewicht. In Verbindung mit den oben beschriebenen Versuchen über die Senkungsreaktion haben wir den Grad der Pseudoagglutination untersucht. Die Blutproben für die Bestimmung der Senkungsreaktion sind 30—60 Min. nach der Mischung mikroskopisch untersucht worden. Bei der höchsten Dextrankonzentration beobachteten wir den stärksten Effekt der bei flüchtiger Betrachtung den Eindruck spezifischer Agglutination machte. Bei näherer Besichtigung liess sich indessen eine primäre Münzrollenbildung wahrnehmen, was auch die Proben mit niedrige-

rem Dextrangehalt bestätigten. Die Abbildungen A—D in Abb. 7 stellen die Pseudoagglutination bei einem Dextrangehalt von 1.0, 0.75, 0.5 und 0.25 % dar. In Konzentrationen bis herauf zu 0.5 % ist dieselbe ziemlich unbedeutend, bei 0.75 % und darüber stark ausgeprägt. Mit Dextran, das in 6 %-iger Lösung eine relative Viskosität von ca. 9 gab, ist der Effekt noch stärker.



A 0.25 % Dextran
B 0.50 % »
C 0.75 % »
D 1.00 % »

Abb. 7. Pseudoagglutination in Menschenblut variierender Dextrankonzentration.

Wie schon hervorgehoben kann man aus in vitro Versuchen nicht mit Sicherheit beurteilen, welche Wirkung diese Steigerung der Senkungsreaktion und Pseudoagglutination der roten Blutkörperchen am Menschen nach grösseren Dextraninjektionen haben kann. Es dürfte doch motiviert sein, mit Ausgangspunkt von diesen Versuchen einige allgemeine Erwägungen anzustellen. Eine schädliche Einwirkung muss auf die erste Zeit nach der Injektion begrenzt sein, während die Senkungsreaktion noch

hoch ist. Die während dieser Zeitspanne herrschende Dextrankonzentration hängt in erster Linie von der Grösse der Infusion ab. Führt man einer Person von 75 kg Körpergewicht und 6 Liter Blut nach einem Blutverlust von 1 Liter 800 ml 6 %-iger Dextranlösung zu, erhält man eine Dextrankonzentration von ca. 0.8 %, die nach zwei Stunden auf 0.6 % reduziert ist. Die Senkungsreaktion dürfte bei ca. 80 mm/1 Std. liegen. An und für sich braucht dieser hohe Wert für die Senkungsreaktion kein Risiko zu bedeuten, aber es ist nicht ausgeschlossen, dass der Dextrangehalt den physikalisch-chemischen Status des Blutes in unvorteilhafter Weise verändert. Diese Erscheinungen hat man schon bei den früheren Versuchen mit Plasmasubstituten wie Gummi arabicum (BAYLISS, 1917), Polyvinylalkohol (HUEPER und Mitarbeiter, 1940) und Polyvinylpyrrolidon (HECHT und WEESE, 1943) beobachtet, und die Veränderungen in der Senkungsreaktion waren in diesen Fällen von derselben Grössenordnung wie die hier beschriebenen. Indessen haben nach den Literaturangaben zu urteilen diese Plasmasubstitute keine Schäden verursacht, die auf eine abnorm starke Pseudoagglutination oder eine erschwerte Zirkulation zurückzuführen wären, wenigstens nicht das Gummi arabicum. Um eine allzu starke lokale Agglutination in den von der Injektionsstelle ableitenden Venen zu vermeiden, haben wir uns die Erfahrung zunutze gemacht, dass eine erhöhte Salzkonzentration die Agglutination aufhebt. Wir haben deshalb das Dextran in unseren späteren Versuchen nicht in physiologischer Kochsalzlösung sondern in 3 %-igem Natriumchlorid gelöst.

Der Kolloiddruck des Dextranhydrolysates.

Der Kolloiddruck wurde in Dextranhydrolysaten von variierender Viskosität und Konzentration in 0.9 %-iger Natriumchloridlösung bestimmt. Die Messungen wurden in Osmometern nach KROGH und NAKAZAWA (1927) mit Kollodiummembranen ausgeführt. Hier soll nur eine Versuchsserie mit dem Präparat Op L 36 mitgeteilt werden, das in 6 %-iger Lösung eine relative Viskosität von 5.0 hatte. Drei Osmometer wurden gleichzeitig mit ein und derselben Lösung gefüllt. Die untersuchten Konzentrationen waren 3.1, 4.7, und 6.2 %. Die Berechnung des Kolloiddruckes geschah folgendermassen. Von der Summe des Manometerdruckes plus Höhe der Wassersäule in den Osmometern wurde der in derselben Weise an einer 0.9 %-igen Natriumchlo-

ridlösung gemessene Druck subtrahiert. In Tabelle 3 sind die Werte zusammengestellt.

Tabelle 3.

Op L 36 Konz. in %	Gemessener Druck in mm H_2O	Mittelwert in mm H_2O	Druck pro % Dextran
6.2	355		
	345	341	55
	325		
4.7	280		
	240	222	47
	145		
3.1	115		
	135	120	39
	110		

Der gemessene Druck ist von derselben Grössenordnung wie derjenige für entsprechende Konzentrationen von Serumeiweiss. Eine 6 %-ige Lösung dieses Dextranhydrolysates gibt einen Druck von 340 mm H_2O und berechnet pro % Dextran nimmt der Druck mit der Dextrankonzentration in ähnlicher Weise ab wie für Serumeiweisslösungen.

Bei der Beurteilung dieses Resultates muss man folgendes berücksichtigen. Die Permeabilität der Kollodiummembran ist nicht dieselbe wie die der Zellmembranen und die polydisperse Dextranlösung enthält eine geringere Menge verhältnismässig kleinmolekularen Materials, welches die Zellmembranen durchtritt. Deshalb muss der Kolloiddruck und die wasserbindende Fähigkeit des Dextrans in den Kapillaren etwas geringer sein als in den Osmometern.

Die Wirkung des partiell hydrolysierten Dextrans bei experimentellem Schock.

Als eine Orientierung über den therapeutischen Effekt der Dextranlösungen haben wir ihre Wirkung auf den experimentell hervorgerufenen Schock an Versuchstieren geprüft. Die verwendeten Präparate waren partiell hydrolysiertes Dextran von demselben Dispersitätsgrad wie in den oben beschriebenen Versuchen. Die Dextrankonzentration betrug ca. 6 % und die Kochsalzkonzentration 0.9 oder 3 %.

Als Versuchstiere dienten Katzen und Kaninchen. Narkosemittel für Kaninchen 0,6 ml Numal »Roche« per kg intravenös, für Katzen 6 ml 25 %-iges Methylurethan per kg Körpergewicht subcutan.

Die Methode für die Blutdruckregistrierung war die für blutige Re-

gistrierung mit Quecksilbermanometer gebräuchliche. Die Atemregistrierung beschränkte sich auf die Atemfrequenz.

Drei verschiedene Schockformen sind studiert worden, der Blutungsschock, der Histaminschock und der traumatische Schock. In sämtlichen Fällen erstrebten wir die höchstmögliche Blutdrucksenkung, die weder letalen Charakter hatte, noch eine sichere Tendenz zu spontaner Steigerung aufwies. Der Blutungsschock wurde durch raschen Aderlass von 40—70 ml Blut durch das Seitenrohr der Carotiskanüle ausgelöst. Der Histaminschock wurde an Katzen mit 0.8 mg Histamin pro kg Körpergewicht hervorgerufen. Der traumatische Schock wurde durch Quetschen der hinteren Extremitäten in tiefer Narkose erzeugt. Im letzteren Falle war die Senkung des Blutdruckes ziemlich schwer zu dosieren, weshalb dieser durch wiederholte geringere Traumata zum gewünschten Niveau gesenkt wurde.

Als Indikator für die Einwirkung der Dextranlösung auf den Schockzustand wählten wir ihre blutdrucksteigernde Fähigkeit. Eine Steigerung des Blutdruckes beim Schock kann mit Kristalloidlösungen allein erreicht werden. Für die so erzielten Effekte, ist es indessen charakteristisch, dass sie weniger ausgeprägt und von mehr oder weniger vorübergehender Natur sind. Besonders bei maximalen Blutdrucksenkungen durch Blutung haben wir nur sehr schwache und vorübergehende Effekte mit physiologischer Kochsalzlösung gefunden. Bei vergleichenden Untersuchungen über den Effekt von Kristalloidlösungen und artifiziellen Plasmasubstituten führte Bayliss (1917) sogenannte Austauschversuche ein. In diesen Versuchen, die sich über recht lange Zeit erstrecken müssen, wird durch wiederholten Austausch eine gewisse Blutmenge mit der zu prüfenden Lösung ersetzt. Entscheidend ist dann, wie viel Flüssigkeit in dieser Weise ersetzt werden kann und wie sich der Blutdruck verhält. Eine Prüfung früherer Versuche, sowie unsere eigenen Versuche sprechen jedoch dafür, dass man den Effekt einer Kolloidlösung teils an ihrer Fähigkeit, den Blutdruck unmittelbar zu steigern und teils an der Dauerhaftigkeit des Effektes messen kann. Wir haben deshalb in sämtlichen Fällen nur eine Steigerung des Blutdruckes ausgelöst, auch wenn diese manchmal durch wiederholte geringere Injektionen erzielt wurde.

Versuche mit Blutungsschock sind an Kaninchen und Katzen ausgeführt worden. In einem typischen solchen Versuch (Versuch 14) wurde eine Katze von 2.8 kg verwendet. Zu Beginn des Versuches wurde der Blutdruck zu 118 mm Hg gemessen. Nach einem Aderlass von 60 ml fiel der Druck auf 15 mm Hg, reduzierte Atemfrequenz und oberflächliche Atmung. Zuerst wurden innerhalb 6 Min. 30 ml 6.5 %-iger Dextranlösung, Präparat 706, in drei Portionen von je 10 ml intravenös gegeben. Hierbei stieg der Blutdruck auf 75 mm Hg. Nach einigen Minuten wurden weitere 30 ml zugeführt, wodurch der Blutdruck sofort auf 100 mm Hg stieg. Im weiteren Verlauf stieg er weiterhin und erreichte

nach 45 Min. den Ausgangswert, wo er dann bis zum Abbruch des Versuches nach weiteren 30 Minuten verblieb.

In einem analogen Versuch mit einem Kaninchen (Versuch 13) von 3.5 kg wurde zu Beginn des Versuches ein Druck von 90 mm Hg registriert. Ein Aderlass von 60 ml Blut bewirkte eine Senkung auf 15 mm Hg. 30 ml 6.5 %-igen Dextrans gab eine unmittelbare Steigerung auf 50 mm Hg, gebesserte Atmung und höhere Pulsamplitude. Nach 12 Min. hatte der Druck ca. 80 mm erreicht und nach weiteren 20 Min. 85 mm, also beinahe den Ausgangswert. Jetzt wurden weitere 15 ml gegeben, die keinen unmittelbaren Effekt erkennen liessen, wohl aber eine langsame weitere Steigerung, sodass der Druck nach 20 Min. 120 mm betrug, also 20 mm höher als der Ausgangswert.

Versuche mit Histaminschock (Versuch H 3) wurden an Katzen ausgeführt. In einem typischen Versuch war der Initialdruck 90 mm Hg. 0.5 mg Histamin senkte den Druck vorübergehend auf 30 mm, worauf er wieder auf 70 mm anstieg. Darauf wurden 1.5 mg Histamin gegeben, die eine Senkung auf 15 mm und Atemstillstand bewirkten. Eine erste Injektion von 30 ml (Präparat 732) steigerte den Druck zu 60 mm Hg in 14 Minuten. Eine weitere Injektion von 20 ml steigerte ihn in 20 Min. auf ca. 100 mm, d. h. höher als den Ausgangswert. Als der Versuch nach weiteren 30 Minuten abgebrochen wurde, bestand dieser Druck noch.

Versuche mit traumatischem Schock wurden an Kaninchen ausgeführt. In einem typischen solchen Versuch (Versuch 6) wurden 3 wiederholte Quetschungen der hinteren Extremitäten vorgenommen. Der Initialdruck war 100 mm Hg und sank auf 30 mm, welcher Druck sich dann konstant zu halten schien. So wurden 20 ml Dextranpräparat nr 709 innerhalb 3 Minuten gegeben und sofort eine Drucksteigerung auf den Normalwert registriert. Während der folgenden 30 Minuten hielt sich der Druck konstant.

Wie die Versuche zeigen, können die verwendeten Lösungen partiell hydrolysierten Dextrans nach intravenöser Zufuhr an Versuchstieren experimentell hervorgerufenen Schock aufheben. Der Effekt tritt besonders durch die blutdrucksteigernde Wirkung hervor. Er setzt unmittelbar nach Beginn der Injektion ein und zeigt sich ausser in der Blutdrucksteigerung auch in einer gebesserten Atmung und gesteigerten Pulsamplitude. Er setzt schon nach Zufuhr geringeren Volumens ein und ist beständig.

Parallelversuche, die hier nicht näher beschrieben sind, wurden mit physiologischer Kochsalzlösung ausgeführt. Ein vollständiger Parallelversuch zu dem hier oben beschriebenen Versuch Nr. 14 ist ausgeführt. Statt Dextran wurde Kochsalz in drei Portionen von je 10 ml gegeben. Jede dieser Dosen gab eine Steigerung von 14.6 bezw. 13 mm Hg, die doch nach weniger als einer Minute zurückging.

Der Effekt, der sich in sämtlichen untersuchten Schockformen als gut erwiesen hat, gründet sich auf die Wasserbindungsfähigkeit des partiell hydrolysierten wie auch unhydrolysierten Dextrans, dessen Makromoleküle die Kapillarmembranen nicht durchtreten. Hierdurch wird das Wasser in den Blutbahnen zurückgehalten und ein ausreichendes zirkulierendes Blutvolumen aufrechterhalten. Auch beim Histaminschock, der durch eine erhöhte Kapillarpermeabilität charakterisiert ist, behält das Präparat hinreichende wasserbindende Eigenschaften. Diese Kenntnis ist auch für die Beurteilung der Anwendbarkeit bei den übrigen Schockformen von Bedeutung. Eine gesteigerte Permeabilität in verbindung mit einer peripheren Gefässparalyse dürfte nämlich im Entstehungsmechanismus des Schocks einen ziemlich wesentlichen Faktor ausmachen.

Vorbereitende klinische Prüfungen.

Da die Tierversuche gut ausgefallen sind, dürfte es motiviert sein, auch klinisch die therapeutische Wirkung bei Infusion von partiell hydrolysiertem Dextran zu prüfen. In solchen klinischen Versuchen haben wir uns bisher darauf beschränkt festzustellen, ob das Präparat in so grossen Dosen vertragen wird, wie es die Schocktherapie erfordert, und welche Konzentrationen im Blut und Harn erreicht werden. Die ersten Versuche sind mit kleinen Dosen von ca. 50 ml ausgeführt und nach und nach gesteigert, so dass nun wiederholte Dosen von 400 ml in einer Anzahl Fälle gegeben sind. Hierbei waren bisher durchaus keine pathologischen Reaktionen zu registrieren. Die Blut- und Harnanalysen in vorbereitenden Versuchen schienen zu zeigen, dass ein Erwachsener einige Stunden nach Infusion von 400 ml 6 %-iger Dextranlösung mit der relativen Viskosität 5 eine Dextrankonzentration im Plasma von ca. 0.4 % und nach 24 Std. ca. 0.3 % hat. Während derselben Zeit wird in den Harn ungefähr ein Fünftel des verabreichten Dextrans ausgeschieden. Für die fortgesetzten klini-

schen Untersuchungen ist eine Zusammenarbeit mit Med. Dr. G. BOMANSSON und Med. Dr. O. WILANDER, Örebro, aufgenommen worden. Die gewonnenen Resultate sollen später mitgeteilt werden.

Zusammenfassung.

Frühere Versuche mit körperfremden Kolloiden als Plasmasubstitut werden relatiert und die Bedingungen, die solche Substanzen zu erfüllen haben, in Kürze zusammengefasst.

Das nicht hydrolysierte, sehr hochmolekulare Dextran ist für Infusionszwecke nicht anwendbar, da es Schäden u. a. an der Leber und den Nieren verursacht.

Die Herstellung und Prüfung partiell hydrolysierten Dextrans wird beschrieben. Partiell hydrolysiertes Dextran ruft bei Infusion an Versuchstieren keine anderen Schäden als eine erhöhte Senkungsreaktion hervor. Das Dextran wird abgebaut und aus den Blutbahnen so schnell eliminiert, dass eine therapeutische Dosis in ca. 4 Tagen entfernt ist.

Lösungen von partiell hydrolysiertem Dextran haben eine günstige Wirkung auf experimentell erzeugten Schock an Versuchstieren. Versuche werden beschrieben, wo das Präparat bei traumatischem Schock, Blutungs- und Histaminschock verwendet wurde.

Eine klinische Prüfung des Präparates ist eingeleitet.

Die Verfasser danken Herrn Professor ARNE TISELIUS für gute Ratschläge bei der Ausführung der Arbeit und den Herren Professoren THE SVEDBERG und ARON WESTERLUND für ihr wohlwollendes persönliches Interesse und für die Vergünstigung, die Arbeit in ihren Laboratorien ausführen zu dürfen.

Wir danken Herrn Med. Dr. NILS GELLERSTEDT für wertvolle Hilfe mit den pathologisch-anatomischen Untersuchungen.

Die klinische Vorprüfung ist durch das Entgegenkommen von Herrn Dr. NILS LINDBORG ermöglicht, wofür wir Ihm herzlichst danken. Herrn Ingenieur S. KLEVÅS danken wir für Assistenz im Laboratorium.

Die Untersuchung ist mit finanzieller Stütze der A.-B. Pharmacia und der Svenska Sockerfabriks A.-B., ausgeführt worden.

Literatur.

BAYLISS, W. M., Proc. Roy. Soc. ser. B. 1917, *89*, 380.
—, J. Pharmacol. 1920. *15*, 29.
DUESBERG, R. und W. SCHWEDER, Klin. Wschr. 1942. 981.
FELIX, K., Verh. Dtsch. Ges. inn. Med. 1940. 387.
GORDON, H., L. J. HOGE and H. Lawson, Amer. J. med. sci. 1942. *204*, 4.
GRÖNWALL, A., und B. INGELMAN, Nord. Med. 1944. *21*. 247.
—, Acta Physiologica Scand. 1944. *7*. 97.
—, and H. MOSIMANN, Nicht veröffentlicht.
HARTMAN, F. W., V. Schelling, H. N. Harkins und B. BRUSCH, Ann. Surg. 1941. *114*. 212.
HECHT, G. und H. WEESE, Münch. med. Wschr. 1943. *90*. 11.
HUEPER, W. C., Arch. Path. 1939. *28*. 510.
—, Amer. J. Pathol. 1939. *15*. 644.
—, J. W. LANDSBERG, und L. E. ESKRIDGE, J. Pharmacol. 1940. *70*. 201.
INGELMAN, B. und K. SIEGBAHN, Arkiv f. Kemi, Mineral., Geol. 1944. *18* B. N:o 1.
JORNS, G., Therap. Gegenwart 1937. *78*. 394.
KLEES, E., Münsch. med. Wschr. 1943. *90*. 29.
KROGH, A. und F. NAKAZAWA, Biochem. Z. 1927. *188*. 241.
LANG, K. und H. SCHWIEGK, Dtsch. Militärarzt 1941, 561. 1942. 379.
MADDEN, C. S., W. A. NOERHREN, G. S. WARAICH und G. H. WHIPPLE. J. exp. med. 1939. *69*. 721.
MADDEN, C. S. und G. H. WHIPPLE, Physiol. Rev. 1940. 20. 194.
MOSIMANN, H. und T. SVEDBERG, Kolloidzschr. 1942. *100*. 99.
NISSLER, K., Klin. Wschr. 1942. 1075.
SVEDBERG, T., Ind. Eng. Chem., Anal. Ed. 1938. *10*. 113—129. (Siehe auch: SVEDBERG, T. und K. PEDERSEN: Die Ultrazentrifuge. Dresden und Leipzig 1940. 366.)

From the Biological Institute of the Carlsberg Foundation, Copenhagen.

Influence of Synthetic Polysaccharid Sulfuric Acid Esters on the Thrombocytes in vivo and in vitro.

By

JØRGEN PIPER.

Received 18 September 1944.

In 1918 HOWELL and HOLT discovered that an anticoagulant substance could be isolated from the liver, and this substance was designated by HOWELL as heparin. During the following years this substance was submitted to a thorough chemical analysis and purification and its physiological properties were investigated (HOWELL, CHARLES and SCOTT, FISCHER, JORPES).

This resulted in a product of a fairly well defined chemical composition which proved completely non-toxic on intravenous injection into animals as well as in man.

Heparin is no ideal anticoagulant as the duration of its effect is too short. Furthermore, it is rather circumstantial and expensive to prepare. For these reasons, attempts have been made during the last two decades to prepare a number of synthetic anticoagulants, but none of them has been found to possess the non-toxicity of heparin.

As finally the chemical composition of heparin was established as a polysaccharid (mucoitin) sulfuric acid ester, BERGSTRÖM (1935) synthesized sulfuric acid esters of other polysaccharides and found that they all possessed some inhibitory action on blood clotting. As to the toxicity of these substances, BERGSTRÖM reported that cellulose sulfuric acid ester is toxic on intravenous injection, producing hemorrhages into the pleura and kidney, and that chondroitin-sulfuric acid ester is tolerated just as well as heparin. As to the toxicity of the other polysaccharid sulfu-

ric acid esters, BERGSTRÖM offers no comment, nor about the cause of the hemorrhages mentioned.

As reported recently, ASTRUP, GALSMAR and VOLKERT (1944) have synthesized highly active chitin, cellulose and starch sulfuric acid esters, and I have investigated the physiological action of these substances. In the present paper some experiments will be reported on the effect of these substances on the thrombocytes *in vitro* and *in vivo*. These studies have revealed an interesting hematological aspect which may be deciding for a probable use of synthetic polysaccharid sulfuric acid esters as substitutes for heparin.

Of the few reports available concerning the effect of this group of substances in the animal organism only the paper by CHARGAFF and OLSON (1937) states that examination of the thrombocytes was carried out in experiments with heparin, cellulose disulfuric acid ester and polyvinyl sulfuric acid ester. These authors state: "The platelet counts were not significantly altered during the course of any of these experiments," — but they fail to record explicitly at which point of time in these experiments the samples of blood were withdrawn for the thrombocyte count. REUSE (1939), working with starch sulfuric acid ester, does not deal with the thrombocytic aspect of the problem.

The thorough investigation of the thrombocytic aspect of the blood under the influence of the polysaccharid sulfuric acid esters originated from the findings in some sedimentation tests carried out with these substances as anticoagulants. Thus it was found that when chitin, cellulose and starch compounds were employed, the sedimentation rate was different from that observed on employment of heparin or citrate; furthermore, the plasma layer did not present the usual opaque appearance, but was clear. For this reason the following experiment was carried out with human blood:

Blood from an arm vein was distributed into 5 tubes, each containing a solution of one of the five anticoagulants to be tried. After spontaneous sedimentation of the blood corpuscles, a drop of the most superficial plasma layer was examined in a counting-chamber where the deposition of the thrombocytes was noted and their number determined. The experiment shows that the chitin, cellulose and starch compounds remove the majority of the thrombocytes from the plasma layer and cause a large part of the remaining to agglutinate (cf. Table I).

Table I.

	4.5 cc blood + 0.5 cc of 3.6 % citrate solution	4.5 cc blood + 0.5 cc of 0.5 % heparin solution	4.5 cc blood + 0.5 cc of 0.5 % K_{20} sol. (chitin comp.)	4.5 cc blood + 0.5 cc of 0.5 % C_{24} sol. (cellulose comp.)	4.5 cc blood + 0.5 cc of 0.5 % S_3 sol. (starch comp.)
Appearance of the plasma layer	milky	milky	almost clear	clear	clear
Thrombocyte count (in thousands)	ca. 300	ca. 300	50—60	ca. 20	ca. 20
Deposition of thrombocytes ..	singly	singly	singly-clusters	singly-clusters	singly-clusters

Several series of corresponding experiments were carried out with various cellulose and chitin compounds, and essentially they gave corresponding results. Sometimes the thrombocyte count was found to be excessively low (only a few thousands) sometimes the reduction was not so marked (a count of 50,000 to 100,000), but in all the cases the values were abnormally low. For further study of these aspects a similar experiment was carried out with a suspension of platelets. One drop of each of the five solutions of anticoagulants was added to a drop of a suspension of platelets placed on the slides. A cover-slip was placed on the top of the mixture and the platelets were observed under the microscope. In the specimens containing a drop of the chitin, cellulose or starch compounds, several of the previously single platelets were seen to aggregate rather rapidly into small heaps, whereas the count remained unchanged. The clustering was most pronounced in the preparations containing the cellulose and starch compounds. The addition of heparin and citrate did not affect the thrombocytes.

After this it may be considered established that the reduction in the thrombocyte count after addition of the synthetic anticoagulants mentioned must be due to a form of agglutination of the thrombocytes, through which the elements sediment in a greater or lesser degree together with the erythrocytes and, thus, are removed from the supernatant layer of plasma.

If a little of the plasma is diluted 1: 10, there is no demonstrable change in the intensity of the clustering of the platelets.

If blood is distributed in a series of tubes containing solutions

of the chitin compound in increasing dilution, the agglutination will take place in all tubes where the blood is still fluid.

An experiment similar to the one with human blood was now carried out with rabbit blood. To a series of tubes containing $^1/_2$ cc of $^1/_2$ % solutions in physiological saline of starch sulfuric acid ester (*S*) and of cellulose sulfuric acid ester (*C*), chitin sulfuric acid ester (*K*) and heparin, together with $^1/_2$ cc of 3.6 % citrate solution, 2.5 cc of rabbit blood, taken from the carotid

Table II.

C_{1115}	: 6—10	thrombocytes,	deposited	singly
K_{50}	: 6—10	»	»	»
S_3	: 6—10	»	»	»
Hep.	: ca. 325	»	»	»
Citrate:	ca. 325	»	»	»

artery is added to each. After cautious mixing and gentle centrifuging for half a minute the thrombocyte content of the plasma layer, together with the deposition of the platelets was examined (cf. Table II. Thrombocytes count in thousands).

Here again, the decreased platelet count obtained on employment of cellulose-, chitin- and starch-compound as anticoagulant has to be explained in the same manner as in corresponding experiments with human blood. No clustering of the thrombocytes is seen in the counting chamber, but the number of platelets is exceedingly small. The proof of an agglutination taking place also in this case is obtained by adding a drop of the cellulose, chitin or starch compound to a drop of the thrombocyte suspension in the citrate plasma, after which the clustering of the platelets may be seen under the microscope.

In order to examine whether the corresponding conditions hold good *in vivo* after the injection of the three compounds mentioned, the following experiments were carried out on rabbits: Small amounts of the substances dissolved in physiological saline are injected into an ear vein. At regular intervals samples of blood (1.4 cc blood—0.6 cc sodium citrate) are taken from veins of the other ear with a paraffined syringe and needle. Citrated blood is employed because the low concentrations of sulfuric acid ester used in such experiments is not always sufficient to prevent coagulation *in vitro*. For, even a very slight commencing coagulation will interfere disturbingly with the experiment.

Experiment 266.

Weight of rabbit: 2,250 g. The normal thrombocyte count is determined prior to the injection. 1 mg K_{49}/kg is injected intravenously. Samples of the blood are taken a few minutes after the injection and then, at intervals of 10—15 min. until the thrombocyte count is normal. Immediately after the withdrawal of each sample of blood, it is centrifuged cautiously for 30 sec., whereafter the thrombocyte content of the plasma layer is examined under the microscope.

Prior to the injection of 1 mg K_{49}/kg: thrombocyte count 480,000.

5	Min.	after	inj:	Thrombocyte	count	15,000,	singly or in small clusters
20	»	»	»	»	»	17,000	singly or in small clusters
35	»	»	»	»	»	10,000	singly or in small clusters
45	»	»	»	»	»	32,000	singly or in small clusters
55	»	»	»	»	»	57,000	singly or in small clusters
66	»	»	»	»	»	ca. 200,000	chiefly in medium-sized clusters
75	»	»	»	»	»	465,000	singly.

The experiment shows that 1 mg K_{49}/kg of chitin sulfuric acid ester acts powerfully on the thrombocytes of the rabbit by lowering the count of singly circulating platelets to a minimum. Gradually, as the experiment proceeds, the count of thrombocytes again rises, the increased number first becoming evident by the appearance of large clusters or flakes of platelets. A little later, 75 min, after the injection, the platelets are singly deposited and may be found in approximately the same number as at the beginning of the experiment.

Experiment 267.

Weight of rabbit: 2,250 g. (Same animal as in the previous experiment). $^1/_2$ mg K_{49}/kg is injected intravenously after determining beforehand the normal count of the thrombocytes.

Prior to the injection of $^1/_2$ mg K_{49}/kg: thrombocyte count 517,000.

5	min.	after	inj.:	Thrombocyte	count	60,000,	situated singly or in small clusters.
15	»	»	»	»	»	35,000	situated singly or in small clusters.
25	»	»	»	»	»	ca. 120,000	chiefly in medium-sized clusters.
35	»	»		»	»	236,000	singly or in small clusters.
45	»	»	»	»	»	ca. 500,000	singly.

In this experiment the dose is half as large as in the previous one. The effect does not last quite so long, and is perhaps not quite so violent. Definitely smaller doses will scarcely have a noticeable effect on the platelets.

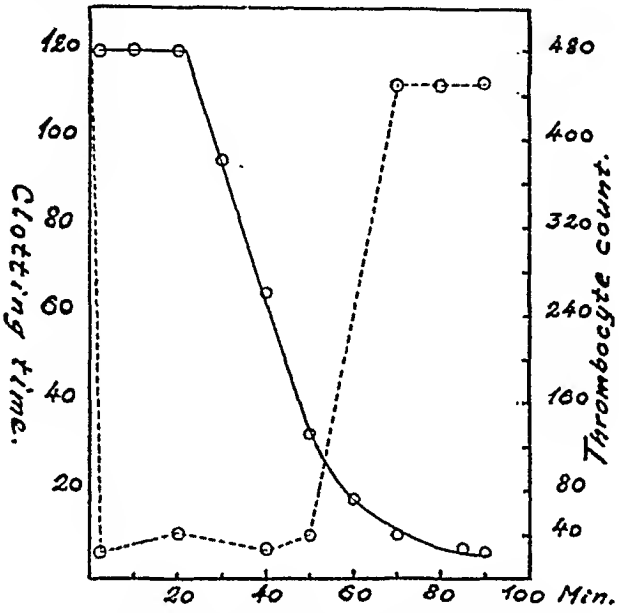


Fig. 1. Effect on the clotting time in minutes and thrombocyte count after intravenous injection of 2 mg K_{49}/kg on a rabbit.

Experiment 265 shows the effect on coagulation as well as on the thrombocytes, after intravenous injection of 2 mg K_{49}/kg. Weight of rabbit: 2,100 g. Under urethan anesthesia the left carotid artery is laid open, a cannule is inserted into the artery and samples of blood are taken every 10 minutes. The result may be read from the curves in Fig. 1. The full-line curve gives the clotting aspects, the stippled curve the thrombocyte counts.

In other experiments, where the clotting time was determined simultaneously with the effect on the thrombocytes, no corresponding good agreement was obtained between the duration of the two widely different effects on the blood. More often the thrombocytic effect was of longer duration, but in a few cases this effect failed to manifest itself at all with doses which proved normally to be effective, and the cause of this failure has not been cleared up fully. As a rule, however, it may be stated that the effect on the thrombocytes is most marked.

The same experiment was carried out on rabbits with cellulose

sulfuric acid ester. The effect of this compound on the thrombocytes *in vivo* is illustrated by the following experiment:

Experiment 270.

Weight of rabbit 2,400 g. Intravenous injection of 1 mg C_{12}/kg. Citrate blood taken from ear vein.

Prior to the injection: 640,000 thrombocytes.

5	min.	after	inj:	Thrombocyte	count	12,000,	situated	singly.
20	»	»	»	»	»	8,000	»	»
50	»	»	»	»	»	4,000	»	»
80	»	»	»	»	»	31,000	singly or in small clusters.	
100	»	»	»	»	»	100,000	singly or in small clusters.	
120	»	»	»	»	»	482,000	singly and a few clusters.	
145	»	»	»	»	»	600,000	situated singly.	

As will be noticed, intravenous injection of cellulose sulfuric acid eter has an even more pronounced effect on the thrombocyte count than has the chitin compound.

Finally, the effect of starch sulfuric acid ester on the thrombocytes was investigated as shown in the following experiment:

Experiment 257.

Weight of rabbit 2,225 g. ½ mg of S_3/kg injected intravenously. The outcome was as follows:

Before	inj.:	614,000	thrombocytes,	deposited	singly.	
2 min.	after	»	39,000	»	singly and in small clusters.	
20	»	»	»	28,000	»	singly and in small clusters.
34	»	»	»	36,000	»	singly or in small clusters.
54	»	»	»	24,000	»	singly and in small clusters.
70	»	»	»	47,000	»	singly and in small clusters.
100	»	»	»	82,000	»	singly and in small clusters.
120	»	»	»	ca. 500,000	»	mostly in large and small clusters.
135	»	»	»	» 500,000	»	mostly in large and small clusters.
155	»	»	»	» 525,000	»	singly — a couple of clusters.

In connection with these experiments, another experiment with heparin will be cited in order to demonstrate that heparin is perfectly indifferent to the thromboeyte eount *in vivo.:*

Experiment 268:

Weight of rabbit 2,250 g. Intravenous injection of 1 mg heparin/kg.

Before inj.:	393,000	thrombocytes,	deposited	singly.
5 min. after »	372,000	»	»	»
35 » » »	373,000	»	»	»

Temporary thrombopenia and intravital agglutination after intravenous injection of some high-molecular substances — *c. g.*, large doses of peptone — have been demonstrated previously. In 1909 ACHARD and AYNAUD reported this interesting hematologieal phenomenon. Soon after intravenous injection of peptone they were able to demonstrate a tremendous fall in the platelet eount to very low values; and the platelets reappeared 15—20 minutes later. ACHARD and AYNAUD assumed that an agglutination took plaee in the liver capillaries where the thrombocytes accumulated. In all the animal experiments this form of thrombocytopenia was aecompanied by leucopenia, and by coma or somnolence. These eoncomitant phenomena disappeared at the return of the thromboeytes to the blood.

PETRI (1926) likewise demonstrated the appearance of thrombopenia shortly after intravenous injection of peptone, but he does not mention the phenomenon of agglutination. He explains the fall in the platelet eount as a "transitory anomaly of distribution". At the juncture when the thrombocytes had reached exeessively low values, no aeeumulation of these elements eould be demonstrated in the blood vessels of the internal organs.

From the experiments here reported it is evident that synthetic sulfuric aeid esters of eellulose, stareh and ehitin, all of which are strong anticoagulants, are able *in vitro* to agglutinate the thrombocytes, so that these elements are removed from the supernatant layer of plasma on the sedimentation of the red blood cells, the plasma thus becoming clear and translucent. *In vivo* the intravenous injeetion of minimal amounts of the substances mentioned produees a marked reduetion in the number of singly circulating thromboeytes. A minimal thromboeyte eount is obtained even as early as two minutes after the injection. The appearance is abnormal, a great many of the platelets agglutinating in small clusters. Gradually, on the reappearance of the plate-

lets, they are found at first to return as lumps or flakes, while later they are distributed singly, and then their number returns to a normal level.

None of the experiments here reported were associated with general symptoms of any character in the animals. When larger amounts of the synthetic substances were injected, however, more or less marked toxic phenomena were observed.

In an attempt to explain the temporary thrombopenia, the following possibilities are to be taken into consideration.

1) After their agglutination the thrombocytes are removed from the plasma layer by sedimentation together with the red blood cells, the normal amount of platelets being found in the samples of blood withdrawn. If this should be the case, the thrombopenia demonstrated is merely apparent.

2) The thrombocytes are present in the vascular system as clusters deposited along the endothelium. In this case, the actual platelet count in a blood sample will be low.

3) The platelets are removed from the blood stream and accumulated as lumps of thrombocytes in the organs serving as a filter for the blood, above all, the lungs, but also the liver and spleen.

4) The disappearance of the thrombocytes from the circulation might be due to destruction of the platelets. But this possibility may be ruled out at once, as the restoration of the platelet count to a fairly normal level may take place within a very short time (about half an hour or a few hours) whereas real regeneration of the same number of platelets will require several days.

In order to exclude the first-mentioned possibility, some experiments were carried out, all giving the same results, on which account it will be sufficient here to cite one of them.

Experiment 340.

Weight of rabbit 2,100 g. Normal thrombocyte count 769,000. A drop of citrate blood was examined under the microscope before the sedimentation had taken place. The specimen showed the thrombocytes to be distributed singly, in an apparently normal number. From another sample of blood, containing 1.5 % solution of cellulose sulfuric acid ester (C_{12}) a drop was likewise examined under the microscope and showed large agglutinates of thrombocytes but the number of thrombocytes was apparently normal. After an intravenous injection of 5 mg C_{12}/kg a sample of blood indicated a considerable decrease of the number of platelets, while no large clusters were seen.

These experiments make it rather probable that the phenomenon observed after intravenous injection involves a true thrombopenia, as an essential part of the platelets are removed from the circulating blood. Probably the clusters of thrombocytes are either deposited on the vascular endothelium or they are sifted from the circulating blood in the various organs.

The agglutination phenomenon here described is widely different from the agglutination of platelets taking place on clotting of the blood. While the agglutination produced by polysaccharid sulfuric acid esters is reversible and produces no change in the appearance of the platelets, the clumping of the platelets taking place on clotting of the blood is irreversible and destructive to the thromboeytes, which thus become firmly united with the fibrin formed.

As to the relation between the effect on the clotting of the blood and the effect on the platelets, no definite rules may yet be laid down. Generally the effect on the clotting time will be of shorter duration than the agglutination of the platelets. In more rare instances, the reverse may take place. Both of these effects are subject to considerable individual variation. Thus in a few animals there is no evidence whatever of the effect on the thrombocyte count with doses in other animals produce a very strong effect; and in other animals the thrombopenia is not so pronounced as observed in the present experiments.

In view of the properties of the polysaecharid sulfuric acid esters here demonstrated, their more or less pronounced toxicity has to be attributed to their agglutinative effect on the platelets, and eonsequently the employment of such eompunds for injection on man has to be avoided. The risk that the aggregates of thrombocytes may give rise to infarction is obvious. Studies on this aspeet of the problem, together with the anticoagulant capacity of these substanees, their exeretion, etc. will be published later. It is a very interesting fact that the organism's own anticoagulant, mueoitin polysulfuric acid ester (heparin) is quite indifferent to the platelets, although it is a eompound of the same type as the above-mentioned.

Whether it will be practicable through a change in the molecular strueture to reduce or abolish the undesirable agglutinating effect on the thromboeytes is a question that is now being studied more thoroughly in experiments with the substanees prepared by KARRER and eollaborators — substances that are claimed to be less toxic than the ones employed here.

Summary.

It is demonstrated that some synthetic anticoagulants of the polysaccharid sulfuric acid ester group (chitin, cellulose and starch sulfuric acid esters) on intravenous injection in rabbits produce an agglutination of the thrombocytes and reduce the number of circulating platelets. This effect is of rather short duration and completely reversible. The toxicity of these substances reported by some authors may be due to the phenomenon demonstrated here.

The mucoitin polysulfuric acid ester (heparin) found in the animal organism does not have the same effect on the thrombocytes.

The present work was carried out with the aid of a grant from "P. Carl Petersens Fond". The rabbits required for the investigations were generously supplied by "Løvens kemiske Fabrik", Copenhagen.

References.

ACHARD, C. and M. AYNAUD, Semaine Méd. 1909, *29*. 169.
ASTRUP, T., I. GALSMAR and M. VOLKERT, Acta physiol. scand, 1944, *8*, 215.
BERGSTRÖM, S., Naturwissenschaften, 1935, *23*, 706.
BERGSTRÖM, S., Hoppe-Seyl. Z. 1936, *238*, 163.
CHARGAFF, E. and K. B. OLSON, J. Biol. Chem, 1937, *122*, 153.
KARRER, P., H. KOENIG, and E. USTERI, Helvet. chim. acta, 1943, *26*, 1296.
PETRI, S., Undersøgelser over Wrights Blodpladeteori. Copenhagen 1926, p. 220—225.
REUSE, J., C. R. Soc. Biol., Paris, 1939, *131*, 834.

From the Finsen Laboratory, Copenhagen.

Concluding Studies on the Conducting Properties of Human Skin to Alternating Current.

By

THOMAS ROSENDAL.

Received 25 September 1944.

Introduction.

Earlier investigations (GILDEMEISTER and his school) have shown that the conduction of human skin to alternating current (a.c.) is capacitive, *i.e.*, here as in the case of a condenser there occurs a phase displacement between alternating voltage and alternating current (voltage after current). Since the phase displacement is less than 90°, the skin corresponds to a condenser with loss. Thus the a.c.-resistance or impedance is complex, and consists of a capacitive and an ohmic component, both of which change at the rise in the frequency of the a.c. The frequency dependence of the a.c.-resistance or impedance of the skin cannot be pictured as a simple electric system consisting of a frequency independent resistance and capacity in series or parallel circuit. It has been impossible to explain the magnitude of the skin capacity by assuming that stratum corneum is a dielectric with a dielectric constant of 7. Since the frequency dependence and loss of the skin capacity show resemblance to the frequency dependence and the loss in the case of a polarization cell, the skin capacity has been regarded as a polarization capacity, and localized to the cell membranes in the living cell layer in the stratum germinativum (GILDEMEISTER and his school, BARNETT 1938).

The demonstration that the a.c.-resistance of the skin almost exclusively is localized to the stratum corneum (ROSENDAL 1940) raises again the question of the nature of the skin capacity, and especially the question of the quality of stratum corneum as a dielectric. The present investigation has for its object a study of this question.

Method.[1]

Object of measurement. The investigations are made on stratum corneum from the heel of cadavers. Here the stratum corneum is about 1 mm thick, and may therefore be dissected off from the underlying tissue and separated almost completely from the stratum germinativum, as verified by microscopy. The a.c.-resistance for frequencies up to 40,000 cycles is partly determined on freshly prepared stratum corneum (area 6 cm^2) and partly on stratum corneum dried for a period of days to constant weight. The a.c.-resistance is moreover determined for a membrane prepared by compressing pulverized, dry stratum corneum. The pulverizing is carried out by trituration of the dry corneous layer in liquid air in a mortar, followed by grinding in a ball mill for several days. The fine, dried stratum corneum powder is then formed under pressure (200—250 kg/cm^2) into a uniform membrane having a thickness of 0.5—0.6 mm. The a.c.-resistance of this membrane has also been determined after soaking with distilled water. Finally, the a.c.-resistance has been determined for a membrane prepared after mixing 300 mg of dry stratum corneum powder with 100 mg of water.

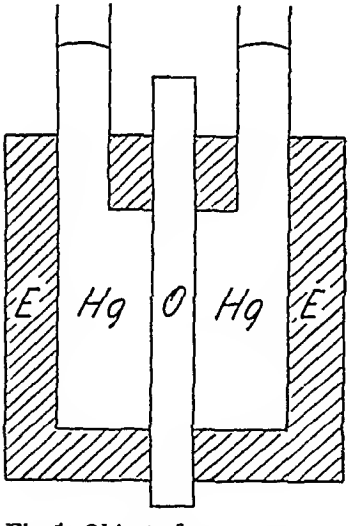


Fig. 1. Object of measurement. O: stratum corneum, or membrane made of compressed, pulverized, dry stratum corneum, the object being placed between 2 mercury electrodes (Hg) in 2 India rubber caps (E).

The object of measurement is placed between 2 mercury electrodes having an area of 6 (in some experiments 3.8) cm^2, as shown in fig. 1. In a few instances the object is placed between 2 parallel circular metal plates having an area of 9.6 cm^2.

Method of measurement. The apparatus used in measuring the complex a.c.-resistance of the stratum corneum or of the membrane consisted either of a GRÜTZMACHER bridge (1934) or a capacity measuring bridge to which the object (fig. 1) is connected. With regard to the first mentioned assembly, reference is

[1] The Laboratory for Telephony and Telegraphy, Danmarks Tekniske Højskole, has most kindly placed its facilities at disposal for this work, and the author wishes here to express his sincere thanks to Professor J. OSKAR NIELSEN. He is also indebted to Mr. R. SVENSSON, Civil Engineer, for valuable advice.

made to an earlier paper (ROSENDAL 1944). Since the GRÜTZMACHER set-up does not permit an accurate determination of the phase angle when the capacity of the object is small (< 2,000 pF), a special bridge has been used in the determination of small capacities. This bridge is built like a Wheatstone bridge with earth impedance balancing, as shown in fig. 2. At each measuring frequency and before connection of standard resistance and standard capacity and of the object of measurement, the bridge is balanced by means of 2 variable resistances and by the differential condenser C, in such a

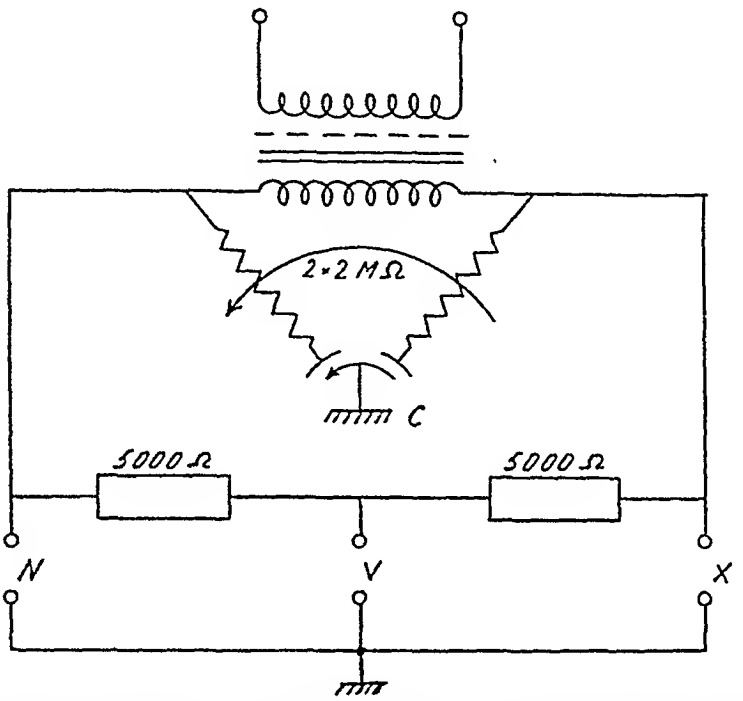


Fig. 2. Diagram of capacity measuring bridge with earth impedance balancing. X: connection to object, V: to a.c.-valve potentiometer, and N: to standard condenser and standard resistance.

way that the a.c.-valve potentiometer used as zero instrument shows 0 as an indication that the earth impedances of the bridge is in balance. The complex resistance of the object is in the bridge assembly determined as a series capacity (C_s) and a series resistance (R_s). The impedance is calculated from $Z = \sqrt{R_s^2 + X_s^2}$ where $X_s = \frac{1}{2 \cdot \pi \cdot N \cdot C_s}$, N being the frequency of the a.c. — and the phase angle from $\tan \varphi = \frac{X_s}{R_s}$. The standard resistance is a non-reactive resistance decade of 100,000 ohms with an error of 0.1 %. In the measurement of greater impedances, calibrated carbon resistances with an error of less than 1 % are used as standard resistance. The standard capacity is a variable condenser of up to 10,000 pF, with an error of 1 %. The source of current is a

heterodyne oscillator with a continuously variable frequency range from 100 to 40,000 cycles. The a.c.-potentials have been less than 2 volts.

When it has been preferred in the majority of the experiments to measure the a.c.-resistance of the stratum corneum between 2 mercury electrodes and not between 2 parallel metal plates, it is because of the error involved in the latter set-up, see table I.

Table I.

Capacity C_s in pF and resistance R_s in ohms as well as the phase angle φ in degrees for 9.6 cm² of stratum corneum dried 8 days in ordinary air; measured between (I) 2 metal electrodes, and (II) between 2 mercury electrodes.

Frequency cycles	I. Metal plate condenser 9.6 cm²			II. Mercury condenser 9.6 cm²			
	C_s pF	R_s ohms	φ degr.	C_s pF	R_s ohms	φ degr.	$\frac{C_s II}{C_s I}$
4000.....	40	138000	82.1	205	108000	71.5	5.13
10000.....	38	43000	84.2	173	42000	74.1	4.55
40000.....	36	6000	86.9	147	8000	79.5	4.1

The experiment shows that the capacity is several times (4—5) lower, is less frequency dependent, and that the phase angle is greater when the corneous layer is measured between 2 metal plate electrodes. The explanation of this circumstance is to be found in the macroscopic structure of the stratum corneum with its gyri and sulci. In set-up I there is only contact between the metal electrodes and the gyri, while the sulci are filled with air. The measuring result is therefore determined by the very small, frequency independent capacity with the phase angle 90° which is represented by the air in the sulci and which is measured in series with stratum corneum's own capacity. In set-up II the mercury fills out the sulci and establishes contact with a larger area; moreover, the distance between the two mercury surfaces is smaller. In this set-up it is solely the dielectric properties of the stratum corneum that determine the capacity measured. Hence it has been used in the majority of experiments described.

Experimental Results.

1) The Significance of the Moisture Content on the a.c.-Conduction of the Stratum Corneum.

In order to elucidate the question of the influence which moisture (water or electrolyte) has upon the a.c.-conduction of the stratum corneum, the impedance and phase angle have been determined at different frequencies for freshly prepared stratum corneum

after thorough wetting of both sides of the corneous layer with $^1/_{10}$ molar KCl-solution, and during drying out in air to constant weight. The results are recorded in figs. 3 and 4 as well as in table II.

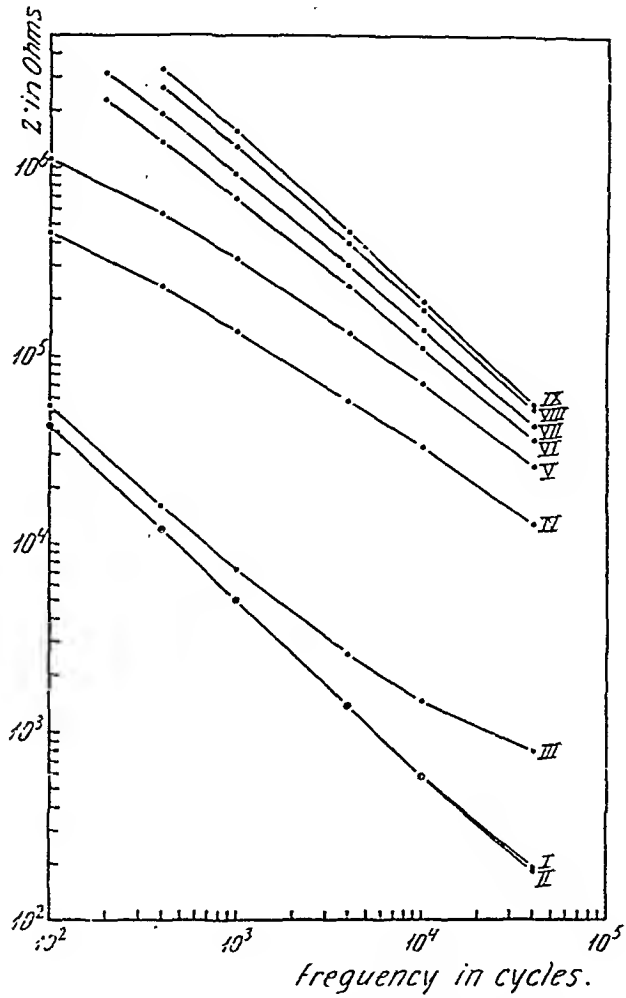


Fig. 3. Impedance values for frequencies up to 40,000 cycles, determined for 6 cm² of the stratum corneum immediately upon preparation (curve I) — after a short-time soaking with $^1/_{10}$ molar KCl-solution (curve II) — and after drying in air for a number of days (curves III—IX). (The Roman numerals in table II correspond to the numbering of the curves in figs. 3 and 4.)

The experiments show practically the same impedance and phase angle for freshly prepared stratum corneum and for the same after soaking with $^1/_{10}$ molar KCl-solution. The frequency dependence of the impedance and the frequency independence of the phase angle at the frequencies 400—10,000 cycles can, in agreement with earlier investigations (ROSENDAL 1940) be expressed by

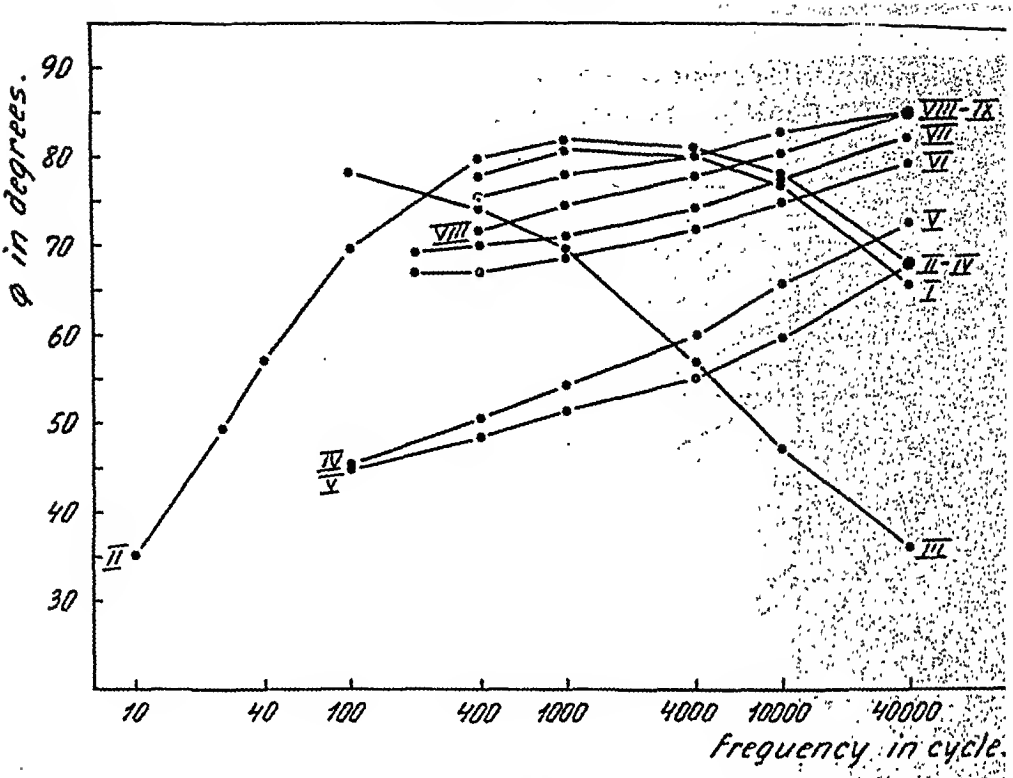


Fig. 4. Phase angle values corresponding to the impedance values for 6 cm² of the stratum corneum in fig. 3.

$Z = k \cdot \omega^{-\alpha}$ and $\varphi = a \cdot \frac{\pi}{2}$ where $\omega = 2 \cdot \pi \cdot N$, a = the slope of the impedance curve and approximately 0.9, and N = the frequency of the a.c. (curves I and II fig. 3 and 4).

The reduction of the phase angle at 40,000 cycles is presumably due to the circumstance that the thick corneous layer has a series resistance which does not decrease with rising frequency, and which at high frequencies, where the reactance $X_s = \frac{1}{\omega \cdot C_s}$ is low, will reduce the phase angle, the latter being determined by $\tan \varphi = \frac{X_s}{R_s}$. The deflection of the impedance curve at 40,000 cycles (curves I and II in fig. 3) is due to the same cause. The experiments further show that the gradual drying out of the corneous layer is accompanied by a very considerable rise in the impedance towards a constant value for the different frequencies, and also by a change in the phase angle. The corresponding changes in the capacity and resistance of the skin as series components

during the gradual drying out are seen in table II. The same table records the weight of stratum corneum as an expression of the degree of drying out.

Table II.

Capacity C_s in pF and resistance R_s in ohms at different frequencies for 6 cm² of the stratum corneum *(I) freshly prepared, (II) after soaking with* $^1/_{10}$ molar KCl-solution, and (III—IX) during gradual drying out in air. The degree of drying is indicated by the weight of the stratum corneum.

Frequency in cycles		100	400	1000	4000	10000	40000	Weight mg
I. Freshly prepared stratum corneum	C_s		34800	32100	29000	27800	23000	570
	R_s		2530	830	243	135	79	
II. Saturated stratum corneum	C_s	41800	33900	31900	29100	28300	23900	
	R_s	14220	2180	720	217	120	65	
III. Drying in air 4 hours	C_s	31100	25900	23300	18300	15250	8560	533
	R_s	10900	4710	2560	1430	975	640	
IV. 24 »	C_s	5000	2310	1500	843	560	336	483
	R_s	315000	152000	85200	33000	16740	4770	
V. 3·24 »	C_s	1920	920	600	338	247	160	477
	R_s	830000	356000	190200	65900	29200	7900	
VI. 4·24 »	C_s		320	252	180	148	113	
	R_s		532000	248500	73000	29500	6650	
VII. 8·24 »	C_s		158	130	104	92	77	460
	R_s		849000	344000	83000	30000	4600	
IX. 13·24 »	C_s		147	122	100	90	77	458
	R_s		857000	339000	77300	27500	6200	

Weight reduction about 20 %

When explaining the cause of the change in Z and φ during the drying out of the corneous layer we shall use an equivalent diagram representing the a.c.-conductance through this layer, see fig. 5.

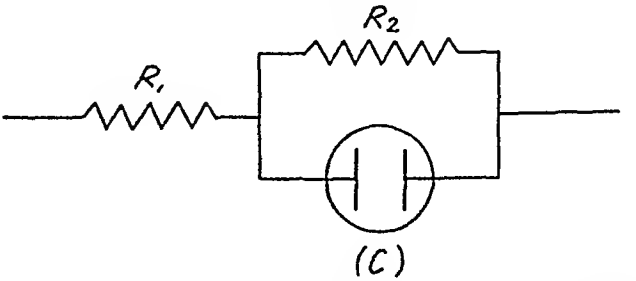


Fig. 5. Equivalent diagram for the a.c.-conduction through the stratum corneum R_1 the series resistance component of the corneous layer, (C) the frequency dependent capacity of the corneous layer, and R_2 the shunt resistance of the capacity.

If we imagine the drying out as an evaporation from the 2 surfaces of the stratum corneum, these areas will, through an array of series resistances of increasing values, be in connection with the deeper lying strata of the corneous layer which are still moist. The rise in the series resistance component R_1, which is frequency independent, will be most pronounced at high frequencies where the impedance is the smallest, and deflect the impedance curve, as seen from curve III, fig. 3. Since the phase angle for the stratum corneum is determined by the equation $\tan \varphi = \frac{X_s}{R_s}$, the rise in R_s will also explain the fall in φ (curve III, fig. 4), likewise at high frequencies.

Gradually as the drying out increases, the corneous layer will more and more approach an ideal dielectric. Correspondingly there is a rise in the impedance of the object (curves III—IX, fig. 3), or a fall in its capacity (table II), while the phase angle approaches 90°, as seen from the curves in fig. 4.

The demonstrated change in Z and φ with the loss of moisture is reversible, a 24 hours soaking of the dry stratum corneum with distilled water restoring the initial values of Z and φ in the frequency range investigated.

2) Investigations on the a.c.-Conduction through a Membrane Prepared from Pulverized Dry Stratum Corneum.

Since it is impossible to determine accurately the area and the thickness of layer of the dried stratum corneum, owing to its gyri and sulci, the experiments mentioned above do not permit a calculation of the dielectric constant (D) of the dry corneous layer. A membrane of known area and with plane and parallel faces was therefore prepared by compressing dry stratum corneum powder, and the capacity and resistance were determined as series components for this membrane at different frequencies. On this basis it is possible to calculate D. The phase angle is determined by $\tan \varphi = \frac{X_s}{R_s}$. The experimental results are recorded in table III.

The experiment shows that the membrane behaves like an almost ideal dielectric with very small frequency dependence of the capacity and with a practically frequency independent phase angle close to 90°.

Inasmuch as the pulverizing of the corneous layer has destroyed the structure of the flat, corneous cells in this layer, experiments

with soaking of the membrane will give information as to the significance of the structure of the corneous layer with respect to the a.c.-conduction found in the case of intact, not dried out stratum corneum.

Table III.

Capacity C_s and resistance R_s as series components, phase angle φ, as well as dielectric constant D for a membrane prepared by compressing dry, pulverized stratum corneum. Area 3.8 cm². Thickness 0.6 mm.

Frequency cycles	C_s pF	R_s ohms	$\varphi°$	D
400.....	54	270000	87.9	9.65
1000.....	53	116000	87.8	9.46
4000.....	52	26000	87.8	9.3
10000.....	51	8000	88.4	9.1

Table IV records a series of determinations of Z and φ for the above mentioned membrane after wetting with a drop of water on a $^1/_2$ cm² large area. The same table contains the values of Z and φ for a membrane prepared by mixing in a mortar 300 mg of dry stratum corneum with 100 mg of distilled water, *i.e.*, a mixture with a moisture content of 25 %, corresponding reasonably well to the loss of moisture from stratum corneum upon drying out. This membrane is soft, and is measured between 2 parallel metal electrodes having an area of 9.6 cm².

Table IV.

Impedance (Z) in ohms and phase angle φ in degrees for a membrane prepared from pulverized dry stratum corneum by compression, after soaking $^1/_2$ cm² with distilled water. Area of membrane 3.8 cm², thickness 0.6 mm.

Frequency in cycles	1000	4000	10000	20000
I Z/φ	730/33	530/22	450/15	430/12
II Z/φ	530/21	476/16.5	440/13.5	420/13.5
III Z/φ	500/20	450/15.5	432/15	424/12

Same for a membrane prepared from pulverized stratum corneum with 25 % water. Area 9.6 cm², thickness 1 mm.

Z/φ	11.5/25	9.5/25	9.5/10

Both experiments show that the soaking of the structureless membrane with distilled water immediately produces a short-circuiting effect with a fall in Z and decreasing phase angle values, gradually as the wetting spreads through the membrane (experimental series I—III). As previously mentioned, stratum corneum that has been soaked through by lying in distilled water 24 hours shows the same a.c.-resistance and phase angle as freshly prepared stratum corneum.

Discussion.

The experiments show that a membrane prepared by compression of pulverized, dry stratum corneum behaves like a good dielectric, the dielectric constant being 9—10, and the lag angle only about 2° in the frequency range 400—10,000 cycles.

The normal moisture (water or electrolyte) content of the corneous layer in situ or immediately after preparation gives rise to a many times greater capacitive conductivity with a lag angle of about 10° in comparison with what is found in the stratum corneum dried in air, values in the frequency range 400—10,000 cycles being 230—300 times greater when the layer is normally moist.

This high capacitive conductivity of the corneous layer when not dried cannot be explained as an interface phenomenon occurring between the mercury electrodes and the electrolyte on the surface of stratum corneum, since the same values of Z and φ have been found by measurement before and after the soaking of a freshly prepared corneous layer with electrolyte. Moreover, other experiments have shown that a short wetting of dried stratum corneum with $^1/_{10}$ molar KCl-solution only raises the capacitive conductivity 1 to 2 times — likewise proof that an interface phenomenon is not responsible. The same experiments show further, that the increase in area and reduction in distance caused by the entry of the electrolyte solution into the furrows and fissures on the surface of the stratum corneum into which the mercury does not reach, is not of sufficient magnitude to explain the high capacitive conductivity of the freshly prepared corneous layer which is found. Hence it must be the moisture (water or electrolyte) content of the corneous layer as a whole that accounts for the high capacitive conductivity in the case of the stratum corneum which has not been dried. However, since the loss of moisture by evaporation is about 20 % of the weight of the corneous layer in the drying-out experiment, and since the dielectric constant of water is about 80, the 20 % moisture can only explain a rise in the capacitive conductivity to about 16 times that of the dry corneous layer.

An earlier paper (ROSENDAL 1940) shows the close similarity between the frequency dependence of the impedance for the stratum corneum in situ and for a series of polarization cells. It moreover finds the phase angle for both systems to be frequency independent and of the same magnitude. Attention is called to the fact, though, that the impedance of the corneous layer is about 600

times greater than that of the polarization cell calculated for the same area. Owing to the resemblance mentioned, and in view of the above cited facts, it seems natural to explain the a.c.-conduction demonstrated as taking place through the stratum corneum by electrolytic polarization. The soaking experiments on the corneous layer and the membrane prepared from pulverized, dry stratum corneum make it seem probable that the polarization must be dependent upon the histological structure of the stratum corneum, and hence must be localized to interfaces in the corneous layer corresponding to its stratified structure.

Summary.

The dielectric constant D of pulverized, dry stratum corneum is found to be 9.1—9.65 for frequencies between 400 and 10,000 cycles. The phase angle is about 88° in the same frequency range.

Determinations of capacity and resistance of 6 cm^2 of the stratum corneum show that it has, immediately after the preparation, a capacitive conductivity which is 230—300 times greater than after it has been dried for several days in air to constant weight, the drying being accompanied by a loss in moisture of about 20 % of the weight of the stratum corneum.

The high capacitive conductivity with a lag angle of about 10° is associated with the moisture (water or electrolyte) content of the corneous layer which has not been dried.

On the basis of the resemblance between the frequency dependence of the corneous layer and that of a series of polarization cells, as well as between the frequency independence of the phase angle for both systems and the magnitude of this angle, the a.c.-conduction through the stratum corneum is explained by electrolytic polarization.

Experiments involving the soaking of a membrane prepared by compression of pulverized, dry stratum corneum make it seem probable that the polarization is dependent upon the stratified structure of the corneous layer.

References.

BARNETT, A., J. Physiol. 1938, *93*, 349.
GILDEMEISTER, M., Handb. norm. pathol. Physiol. 1928. *8:* 2, 657.
GRÜTZMACHER, M., Telegraphen- und Fernsprech-Technik 1934, *23*, 27.
ROSENDAL, TH., The conducting properties of the human organism to alternating current, Diss., Copenhagen 1940.
ROSENDAL, TH., Acta physiol. scand., 1944.

From the Chemical Laboratory, Atlas Ltd., Copenhagen and the Biochemical Institute, Aarhus University, Denmark.

Some Remarks on the Kinetics of the Carotene Loss in Dried Vegetables.

By

EIGIL ETTRUP PETERSEN and FRITZ SCHØNHEYDER.

Received 26 September 1944.

In artificially dried and stored vegetables, which in an increasing degree are employed as foodstuffs, a loss of carotene can be ascertained. This loss is generally supposed to be due to oxydases. Only a few papers discuss the problem of the course of the loss-curve of carotene in dried vegetables during storage. KANE, WISEMAN and CARY (1937) have found that alfalfa meal, kept in a dark, not heated loft lost 3 per cent of carotene on an average per month when the outer temperature was 7 to 19° C, and 21 per cent per month, when the outer temperature was above 19° C. TAYLOR (1934) finds at a temperature not stated a carotene loss of 50 to 67 per cent in alfalfa during storage 3 to 7 months. BIELEFELDT (1942) has investigated the stability of carotene of alfalfa at different water contents. He stated that under good storage conditions there is a carotene loss of 30 to 60 per cent during 9 months. The above mentioned authors have thus all calculated the percentage loss of carotene during some definite period and have not been appreciably interested in the kinetics of the process.

In the following pages it will be shown that when the temperature is kept constant and the water content only varies within rather narrow limits the logarithm of the carotene content y in dried vegetables will vary linearly with the duration t of storage. The loss of carotene thus proceeds after an exponential function $y = e^{\alpha + \beta t}$ according to a first-order reaction scheme. Consequently the carotene loss of these materials at different conditions

can be characterized by the time of half decomposition, $t_{0.5}$. For carotene in carrots, kale, spinach and alfalfa $t_{0.5}$ under identical outer conditions will be mentioned. The relationship between $t_{0.5}$ for carotene and water contents of materials is further elucidated in the case of carrots.

Material and Methods.

Carrots, kale, spinach and alfalfa have been investigated. The quite fresh vegetables are carefully washed in cold water, minced and dried in a current of air at 90° C. Time of drying about 40 min. The dried materials are homogenized and placed in desiccators over saturated salt solutions in a thermostat at 25° C. At suitable intervals samples are taken for the determination of carotene. The amount of carotene is given as mg carotene per 100 g of dry matter.

Determination of carotene. 1/4 to 1 g of material is weighed out and placed in a 200 ml flask. 10 ml of KOH solution (125 g KOH + 100 ml water) + 70 ml alcohol, 96 per cent, are added. The flask is heated for 3 quarters of an hour on a steam-bath with reflux condensor. After cooling the solution is extracted repeatedly with 50 ml benzine (boiling point below 70° C). The benzine extract is decanted into a separating funnel containing 100 ml water. After shaking the water phase is tapped into a separating funnel containing 25 ml benzine, after which the procedure is repeated until the benzine does not take up any colour from the water phase. The benzine extract is now fractionated by repeated shaking with 50 ml "methanol mixture". The total methanol mixture is shaken in a separating funnel with 25 ml "benzine mixture" until the benzine mixture remains colourless after shaking. The benzine mixture employed here is added to the primary benzine extract which contains the main part of the carotenoids. The "methanol mixture" (methanol saturated with benzine) and "benzine mixture" (benzine saturated with methanol) are prepared as follows: 2 l benzine, 2 l methanol and 125 ml water are mixed. When the two layers have separated sharply, the bottom layer ("methanol mixture") is tapped off. Top layer = "benzine mixture". — The total benzine extract is washed three times with 100 ml of water, the extract then being dried with sodium sulphate. The dried extract is filled into a 150 to 200 ml measuring flask and pure benzine is added to the known volume. The carotene concentration of the solution is measured by means of Pulfrich's photometer using filter S. 45. 5 readings are taken on each side using two different cuvette lengths. From the readings $E_{1\ cm}$ is computed. Let a be the amount of dried material weighed out, b volume of benzine extract in ml and c percentage of

dry matter. The amount of carotene per 100 g dry matter is then equal to $\frac{E_{1\,cm} \cdot 401 \cdot b \cdot 100}{c \cdot a}$ mg. In principle this method corresponds to the authorized standard method given by the Danish ministry of agriculture for the determination of carotene in alfalfa meal. — On account of the restrictions with regard to benzine for extraction the work has chiefly been carried out with single determinations. A greater series of double determinations has shown that the variation coefficient of a single carotene determination is about 5 per cent.

The great majority of the carotene determined is β-carotene. By chromatographic methods KUHN and BROCHMANN (1933) have found that 85 per cent of the carotene in carrots is β-carotene, whereas only 15 per cent is α-carotene and only 0.1 per cent γ-carotene. KUHN and LEDERER (1931) have shown that spinach does not contain α-carotene. BIELEFELDT (1942) has found 96 to 98 per cent of the carotene in alfalfa to be β-carotene. In 10 g of fresh kale, containing 32 mg, β-carotene per kg no α- and γ-carotene could be demonstrated[1]).

Determination of the water content. A weighed amount of material is placed in a thermostat at 105° C. After standing for 24 hours constant weight was reached. The water content in per cent was calculated from the weight before and after drying. The variation coefficient of the water content determination was about 7 per cent.

Desiccators. To adjust the materials to a suitable and relatively constant degree of moisture they are placed in desiccators in a way similar to the one used by BIELEFELDT (1942). The different desiccators contain the following salts in pure state or in equilibrium with their corresponding saturated solutions (Table I). The water content

Table I.

Desiccator No.	Salt
1	Phosphorpentoxyde in substance
2	Potassium acetate in sat. solution
3	Zinc nitrate in sat. solution
4	Calcium nitrate in sat. solution
5	Ammonium chloride in sat. solution

of the different vegetables vary to some extent at the same degree of moisture. It ought to be mentioned that equilibrium occurred slowly in the desiccators 1 and 5. The desiccators were placed in a thermostat at 25° C + 0.5° C.

Results.

With each of the materials of dried carrots, kale, spinach and alfalfa two determinations of the half decomposition time for

[1]) The authors are indebted to the Laboratory of *Aarhus Oliefabrik* for this determination.

Table II.

8 Experiments on the Loss of Carotene in Dried Vegetables

Carrots						Kale					
Exp. 1			Exp. 2			Exp. 3			Exp. 4		
1	2	3	1	2	3	1	2	3	1	2	3
0	88.4	51.6	0	88.0	48.9	0	93.0	38.8	0	92.1	34.8
33	88.3	27.1	20	86.9	37.1	35	94.4	36.9	54	91.2	26.5
44	87.9	26.5	33	86.2	32.9	95	94.3	21.1	82	92.2	25.7
53	88.7	20.1	44	86.7	27.1	124	94.1	18.3	113	92.0	20.1
67	88.5	15.5	53	86.6	24.7	154	94.0	17.9	142	92.0	18.3
						184	94.0	13.3	170	91.8	17.2
						204	94.1	11.9	255	90.5	11.4

Spinach						Alfalfa					
Exp. 5			Exp. 6			Exp. 7			Exp. 8		
1	2	3	1	2	3	1	2	3	1	2	3
0	94.6	43.1	0	92.8	33.1	0	93.6	23.8	0	92.7	18.2
62	94.7	32.7	59	92.4	27.4	39	94.7	18.6	59	92.5	13.5
90	95.2	28.9	83	93.0	26.5	89	94.3	16.3	90	92.9	12.7
121	95.1	29.1	110	93.4	24.4	121	94.3	15.2	118	92.5	11.5
173	95.9	24.8	189	92.7	19.1	149	94.2	13.7	168	92.4	8.2
191	94.7	21.9	195	92.7	18.9	223	94.7	9.7	202	92.4	8.0
272	95.5	19.5	279	92.5	12.5	233	94.7	8.5			

Column 1: Duration of experiment in days.
» 2: Dry matter in per cent.
» 3: mg carotene per 100 g dry matter.

carotene at different water content will be reported in detail. Table II gives percentage dry matter and carotene for the different experiments. Table III gives the average water content during the experiments, the coefficient β for the regression of the logarithm of the carotene contents at on the time of storage, calculated by the method of least squares, the variation coefficient of β, and $t_{0.5}$ calculated from the relation $t_{0.5} = \frac{0.3010}{\beta}$. Column 8 shows the numbers of degree of freedom f and column contains $\Sigma'\Sigma\Delta^2 = 0.024372$ (Δ = difference between log carotene observed and log carotene calculated from the theoretical straight line). S_y (the standard error of estimate of log carotene) $= \sqrt{\frac{\Sigma'\Sigma\Delta^2}{\Sigma f}} = 0.0264$. Antilog $0.0264 = 1.063$; this means that

Table III.

Exp. No.	Dried material	Desiccator No.	Average water content per cent	β	V per cent	$t_{0.5}$ days	f	$\Sigma\Delta^2$
1	carrots	2	11.6	0.00764	8.1	39.4	3	0.002995
2	»	3	13.1	0.00560	4.5	53.8	3	0.000331
3	kale	2	6.9	0.00260	7.7	115.8	5	0.006839
4	»	4	8.3	0.00188	4.9	160.0	5	0.001784
5	spinach	2	4.9	0.00124	9.5	242.7	5	0.003449
6	»	4	7.2	0.00145	6.8	207.6	5	0.002648
7	alfalfa	2	5.6	0.00177	7.3	170.1	5	0.003931
8	»	4	7.4	0.00183	8.1	164.2	4	0.002395
								$\Sigma'\Sigma\Delta^2$ 0.024372

the standard deviation of a single determination of carotene is about 6 per cent, and as previously mentioned the variation coefficient of a single determination is about 5 per cent it will be seen that the deviation, caused by variations in experimental conditions, has been very small.

As the decomposition process is the result of the influence of the oxygen in air, and as the carotene has varied in concentration in the materials, the oxygen must have entered in the process with a constant potential, therefore all the carotene in the dried products must have been equally accessible to the influence of the air. Hence it seems rational to calculate the half decomposition time ($t_{0.5}$) of the loss in carotene content and use this value as a measure of the stability of carotene under varying conditions. Experiments which are not reported in this paper have shown that more than 90 per cent of the carotene decrease follow a first-order reaction scheme.

It is seen from Table III that under the mentioned storage conditions the stability of carotene in dried carrots is materially lower than in dried kale, spinach and alfalfa. The greatest half decomposition time was found for spinach.

Table IV shows the result of some investigations on the relation between $t_{0.5}$ for carotene and the water content in dried carrots. The experiments are in the table arranged according to increase of water content. The different average water content of dried carrots in the same desiccators is due to the fact, that the materials have been dried to different water content during the process of drying. Moreover it is a question of materials that have been dried at three different times in the course of one year. It

Table IV.

Exp. No.	Desiccator No.	Average water content per cent	$t_{0.5}$ days
17	1	7.0	5.6
12	1	8.9	21.9
13	2	8.9	27.7
9	1	10.1	25.2
14	3	10.9	26.3
1	2	11.6	39.4
15	4	11.9	38.6
2	3	13.1	53.8
16	5	13.7	49.6
10	5	15.8	53.9
11	5	18.0	62.2
18	5	21.5	47.5
19	5	23.7	39.5

appears from Table IV that there is a close relationship between the water content of the materials and $t_{0.5}$. At small water content the stability is very poor. At about 18 per cent of water the stability is greatest. BIELEFELDT (1924), who has determined the percentage loss of carotene in alfalfa at different times, has found the greatest stability of carotene at degrees of moisture of 42 and 75 per cent corresponding to a water content in alfalfa of about 7.5 and 13 per cent respectively. Between these values and below 7.5 per cent water in alfalfa BIELEFELDT found low stability of carotene. According to our investigations two optima of stability have not been found for dried carrots, and optimum of stability is found at considerably greater water content than in alfalfa. From a series of BIELEFELDT's experiments it seems possible to draw the same conclusions with regard to the kinetics of the reaction as from our experiments. If the loss of carotene in some of BIELEFELDT's experiments disagrees with a reaction of first order it is no doubt because the conditions of storage have not been so constant as in our experiments.

Summary.

In dried carrots, spinach, kale and alfalfa, stored at constant temperature and in desiccators with constant degree of moisture in order to keep the water content of the dried materials approximately constant at different levels, it is shown that the

carotene loss proceeds according to a first-order reaction scheme; hence it seems rational to calculate the half decomposition time for carotene and use this value as a measure of the stability of carotene under varying conditions. It is shown that under identical conditions the half decomposition times for the dried green vegetables: spinach, kale and alfalfa are of the same order of dimension, apparently greatest however, for spinach. $t_{0.5}$ for carotene in carrots is materially smaller than for the above mentioned green vegetables. In dried carrots the stability of carotene depends greatly upon the water content, the greatest stability being found at a water content of about 18 per cent.

The authors are grateful to "Tuborgfondet" for a grant in aid of this research.

References.

BIELEFELDT, J., Studier over Karotin, Disp. København, 1942.
KANE, E. A., H. G. WISEMAN and C. A. CARY, J. agric. Res. 1937. *55*. 837.
KUHN, R., and H. BROCKMANN., Ber. dtsch. Chem. Ges. 1933. *66*. 407.
KUHN, R., and E. LEDERER, Ibidem 1931. *64*. 1349.
TAYLOR, M. V., New Jersey Agricult. 1934. *6*. 3.

From the Biochemical Institute, Aarhus University, Denmark.

On the Affinity of Pig Pancreas Lipase for Tricaproin in Heterogeneous Solution

By

FRITZ SCHØNHEYDER and KIRSTEN VOLQVARTZ.

Received 26 september 1944.

In a recent paper (SCHØNHEYDER and VOLQVARTZ, 1944) it was shown, that the affinity of pig pancreas lipase for triacetin, tripropionin, tributyrin, trivalerin and tricaproin in homogeneous solution was extremely low, direct proportionality being found between substrate concentration and the initial velocity of the enzymatic hydrolysis at the same enzyme concentration. Apparently investigators have only been slightly interested in measuring the affinity of lipase for a triglyceride, which is present in undissolved state. WEINSTEIN and WYNNE (1935—36) have, however, made an attempt with tripropionin, but these authors have not taken into consideration, that the kinetics of the hydrolysis are different in homogeneous and heterogeneous solutions. They apply the equations which can be derived for a homogeneous system to heterogeneous systems. In the case where the solubility of a triglyceride is exceeded the triglyceride is present in two phases, an aqueous and a lipid phase. When lipase is added to such a mixture the reaction between enzyme and substrate will take place both in the aqueous phase and in the surface layer between the aqueous and the lipid phase. In order to determine the affinity of lipase for undissolved triglyceride it is thus necessary to know the ratio between the amounts of dissolved and undissolved triglyceride and the magnitude of the initial area of the lipid phase at varying amounts of substrate.

In the following paper our experiments on the determination of the affinity of pig pancreas lipase for tricaproin in heteroge-

neous mixtures are given. This triglyceride has been chosen, because it is only slightly soluble in water and easily emulsified by the technique employed.

Experimental.

The determination of the enzymatic velocity of hydrolysis was carried out as in our previous work (SCHØNHEYDER and VOLQVARTZ, 1944), the amount of acid liberated during the reaction being neutralized by adding dropwise 0.04 n NaOH, keeping pH almost constant. pH was measured by means of a protected glass electrode and a Radiometer potentiometer. It ought to be mentioned, that in the experiments reported in this paper, intensive shaking of the reaction mixture was used (250 double oscillations per min.), carbon dioxide-free air being bubbled through the mixture. The system employed consisted of tricaproin in water, to which was added 5 mg per cent sodium taurocholate, 80 mg per cent $CaCl_2$ and Michaelis' veronal buffer. The total volume of the reaction mixture was generally 250 cc. At the lowest substrate concentrations volumes up to 750 cc were used. Before the beginning of the experiment the pH of the mixture was adjusted to about 7.5. Addition of activating substances was found necessary to obtain reproducable velocities of hydrolysis. The sodium cholate concentration chosen was rather low owing to the difficulty of getting larger amounts of this material. In the case of $CaCl_2$ the optimal concentration was used. 2 cc of veronal buffer was used per 250 cc of reaction mixture. At the lowest substrate concentrations relatively smaller amounts were used. Temp. 30° C $\pm$ 0.5° C. The tricaproin was added to the reaction mixtures from calibrated capillary pipettes.

The *initial reaction velocity* v was calculated graphically by plotting number of drops of 0.04 n NaOH (1 cc = 44 drops) against time in min. During the beginning of the reaction the curve was always found to be linear and v was computed as number of drops added during the first 10 minutes of reaction. At the lowest substrate concentrations only one to three additions of NaOH could be used, as the reaction here showed a tendency to stop within a short time.

The tricaproin used had the molecular weight 407 and $d_4^{20} = 0.981$. The solubility was determined by preparing a series of emulsions with decreasing amounts of tricaproin per unit of volume. Samples of emulsions were examined under the microscope in a Helber-Glynn's counting-slide by a magnification 1 : 400. The first mixture in which fatty globules could not be observed, in spite of careful examination, contained 1.2×10^{-3} mols per liter caproin.

Pig pancreas lipase was prepared according to the method of WILLSTÄTTER and WALDSCHMIDT-LEITZ (1923). In homogeneous system a preparation E, which had been prepared by diluting the original extract A with glycerol 1 : 10 by weight was used. In heterogeneous system a preparation K = A diluted 1 : 80 by weight with glycerol was used.

The Area of the Lipid Phase.

When calculating the surface of the lipid phase from the amount of tricaproin added a correction for the amount of triglyceride dissolved must be introduced. The rest is present as emulsified fatty globules and the calculation of the total surface of the particles is illustrated by the following example.

Table I.

Characteristic and Computation of the Ratio $\frac{A}{V}$ for 0.0738 cc Tricaproin Emulsified in 250 cc of Solution at 30° C. Shaking: 250 Double Oscillations per Min. Emulsion No. V.

D mm photo	Number of drops with diameter = D	Number of drops in per cent with diameter ≦ D	log D	Probit	mm²	mm³
0.40	1	0.7	0.602—1	2.54	0.1600	0.0640
0.45	1	1.4	0.653—1	2.80	0.2025	0.0911
0.50	11	8.9	0.699—1	3.65	0.2500	0.1250
0.56	5	12.3	0.748—1	3.84	0.3136	0.1756
0.60	2	13.7	0.778—1	3.91	0.3600	0.2160
0.64	3	15.8	0.806—1	4.00	0.4006	0.2611
0.66	8	21.2	0.820—1	4.20	0.4356	0.2875
0.68	2	22.6	0.833—1	4.25	0.4624	0.3144
0.71	4	25.4	0.851—1	4.31	0.5041	0.3579
0.75	11	32.9	0.875—1	4.56	0.5625	0.4219
0.78	1	33.6	0.892—1	4.58	0.6084	0.4746
0.80	11	41.1	0.903—1	4.78	0.6400	0.5120
0.83	5	44.5	0.919—1	4.86	0.7056	0.5927
0.86	2	45.9	0.935—1	4.90	0.7569	0.6585
0.90	14	55.5	0.954—1	5.14	0.8100	0.7290
1.00	19	68.5	0	5.48	1.0000	1.0000
1.10	7	73.3	0.042	5.62	1.2100	1.3310
1.13	3	75.4	0.053	5.69	1.2769	1.4429
1.20	7	80.1	0.079	5.85	1.4400	1.7280
1.25	4	83.0	0.097	5.95	1.5625	1.9531
1.29	4	85.7	0.111	6.07	1.6641	2.1467
1.40	3	87.7	0.146	6.16	1.9600	2.7440
1.45	1	88.5	0.161	6.20	2.1025	3.0486
1.50	2	89.9	0.176	6.28	2.2500	3.3750
1.56	2	91.1	0.193	6.35	2.4336	3.7964
1.60	1	91.8	0.204	6.39	2.5600	4.0960
1.63	1	92.5	0.212	6.44	2.6569	4.3307
1.66	1	93.3	0.220	6.50	2.7556	4.5743
1.75	3	95.3	0.243	6.68	3.0625	5.3594
1.90	2	96.8	0.279	6.85	3.6100	6.8590
2.00	2	98.1	0.301	7.08	4.0000	8.0000
2.10	1	98.8	0.322	7.26	4.4100	9.2600
2.20	1	99.5	0.342	7.58	4.8400	10.6480
2.40	1	100.0	0.380	—	5.7600	13.8240
	146		0.303—6		162.2988	212.5153

Emulsion V. 0.0750 cc tricaproin was added dropwise to 250 cc of solution at 30° C. The lipid phase = 0.0750—0.0012 = 0.0738 cc. The mixture was shaken for 10 min. 250 double oscillations per min. Then samples of the emulsion were placed in a Helber-Glynn counting slide (depth 20 μ). The slide was immediately placed under the microscope and illuminated from the side. Ocular: No. 10. Objective: No. 40. By means of a Leica camera a series of photographs was taken of the fatty globules, which could easily be recognized. To get a measure of the degree of magnification, a graduated mm was photographed, 1 mm photo corresponding to 2.475 μ real. The degree of magnification is thus 404 times. Now all the particles photographed were counted and measured, (146 particles in 15 photos). — By the smallest amounts of tricaproin per unit of volume not more than two particles could be found in each field of vision. In case of greater amounts 10 to 15 particles were counted in each field of vision. None of the particles were found to be flattened. — Table I contains a characteristic of the emulsion examined and numerical material for the computation of the ratio between the total surface of the fatty particles and their total volume $\left(\frac{A}{V}\right)$. In the colums I and II the diameters D are given according to the size of D (in mm photo) and the number of drops with diameter D. When a histogram is drawn with D as abscissa (interval width 0.40 mm) and n as ordinate, it is seen that the distribution is skew (positive). On the other hand when the histogram is drawn with log D (column IV) as abscissa (interval width = 0.15) and n as ordinate a normal frequency distribution curve is obtained. When the probit method is employed (cf. columns III and V) the points are excellently grouped around a straight line (log D = log $D_{50\,\%}$ + σ (probit —5)). log $D_{50\,\%}$ and σ were calculated by the method of least squares. For emulsion V the following parameters were found: log $D_{50\,\%}$ = 0.942 — 1, σ = 0.165, corresponding to a geometric mean diameter of 2.16 μ. The calculation of the surface corresponding to a known volume by means of the statistically calculated distribution of the diameters would involve a difficult integration, wherefore the ratio $\frac{A}{V}$ has been calculated from all the individual diameters measured. The ratio between total surface A and total volume V (all lengths in cm) is

$$\frac{A}{V} = \frac{\pi\, D^2}{\frac{\pi}{6}\, D^3} = \frac{6 \times 10^4}{2.475} \times \frac{(\text{mm photo})^2}{(\text{mm photo})^3} = 2.422 \times 10^4 \times F$$

F = 0.764 is calculated from the figures in columns VI and VII in Table I. $\frac{A}{V} = 1.89 \times 10^4$. — In a similar way as in the case of emulsion V the ratio $\frac{A}{V}$ has been determined for 6 other emulsions. *Fig. 1* shows the relation between probit and log D for these emulsions. The

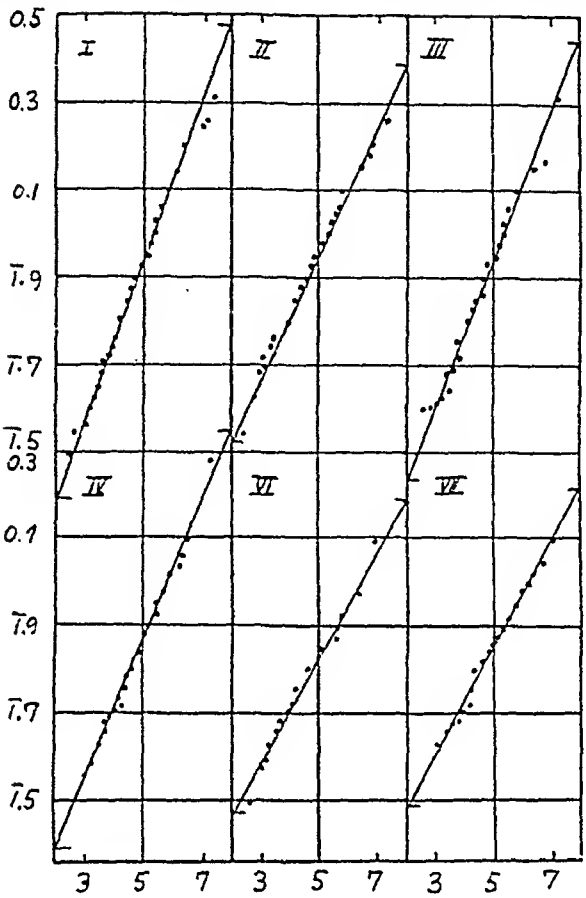


Fig. 1. Relation between probit and logarithm of diameter of tricaproin particles in emulsions I, II, III, IV, VI and VII. Abscissa probit; ordinate log D.

Table II.

Characteristic and Computation of the Ratio $\frac{A}{V}$ *for 7 Different Tricaproin Emulsions with Increasing Tricaproin Content.*

Number	cc Tricaproin added to 250 cc solution	Number of particles counted	log $D_{50\,\%}$	σlog D	Σ mm²	Σ mm³	F	Percentage deviation from $\bar{F}$
I	0.00625	65	0.955—1	0.123	66.04	74.70	0.884	+ 2
II	0.0125	91	0.973—1	0.121	104.99	129.04	0.814	— 5
III	0.0250	86	0.939—1	0.160	88.36	105.39	0.838	— 3
IV	0.0500	98	0.866—1	0.167	77.96	85.95	0.907	+ 5
V	0.0750	146	0.942—1	0.165	162.30	212.52	0.764	—12
VI	0.1000	126	0.849—1	0.143	86.85	88.36	0.983	+14
VII	0.1250	200	0.872—1	0.178	168.99	196.91	0.858	— 1

straight lines indicate, as in the case of emulsion V (Table I), a normal distribution with regard to log D. In Table II it is seen that the average values of the logarithm of the diameters of the tricaproin particles for the 7 emulsions agree closely, and the standard deviations of log D are of the same order of dimension. It is further seen, that the ratio between surface and volume is very nearly constant, as there is no trend in F to increase or decrease by increasing amounts of substrate. It seems thus justified to use a constant conversion factor: $2.422 \times 10^4 \times F$ in the range examined. The conversion factor $2.422 \times 10^4 \times 0.864 = 2.09 \times 10^4$ has the variation coefficient 8.1 per cent and the standard error is 3.0 per cent. By multiplication of non dissolved substrate in cc by the conversion factor the initial surface in cm^2 under the experimental conditions given is obtained.

Results.

In Table III are given the experiments which make the basis of the calculation of the affinity of pancreas lipase for tricaproin in a heterogeneous system. In all 34 experiments have been carried out at 12 different substrate concentrations. At substrate concentrations $\geqq 0.0125$ cc tricaproin in 250 cc, the volume of the system was 250 cc. At lower concentrations the reaction volume was increasing, though not above 750 cc. In experiments 1—7, where the system is homogeneous the lipase preparation E, which was 8 times stronger than K was used. The figures in column IV are calculated as the difference between added and dissolved tricaproin multiplied by 2.09×10^4. In column V are given the amounts of enzyme, in all cases converted to 250 cc reaction mixture. In columns VI and VII the ratio $\frac{v_{uncorr}}{g_K}$ and the average value of these ratios at each substrate concentration are to be found. v_{uncorr} is the velocity measured in the experiment. For the three homogeneous solutions proportionality is found between v and substrate concentration at the same amount of enzyme in agreement with earlier findings (SCHØNHEYDER and VOLQVARTZ, 1944). By transition to the heterogeneous systems $\frac{v_{uncorr}}{g_K}$ rises rapidly and approaches a maximal value very quickly. The double determinations agree fairly well and by varying the enzyme concentrations at the same amount of substrate it is found, that in the range where the experiments are carried out, there is proportionality between v and amount of enzyme. No spontaneous hydrolysis was observed in the mixtures examined. The figures

Table III.

The Reaction Velocity in Relation to the Substrate Surface.

Exp No.	Tricaproin per 250 cc g	Dissolved tricaproin $m \cdot 10^5$	Surface of lipid phase in 250 cc cm^2	Enzyme per 250 cc g	$\frac{v_{uncorr}}{g_K}$	$\frac{v_{uncorr}}{g_K}$	$\frac{v_{uncorr}}{g_K \cdot m \cdot 10^5}$	$\frac{v_{calcul}}{g_K}$
1	0.00046	0.455	0	5.00E	0.262	0.262	0.576	
2	0.00092	0.91	0	2.50E	·0.491			
3	0.00092	0.91	0	2.50E	0.507	0.494	0.542	
4	0.00092	0.91	0	5.00E	0.491			
5	0.00092	0.91	0	5.00E	0.485			
6	0.00115	1.13	0	0.50E	0.55	0.63	0.557	
7	0.00115	1.13	0	0.50E	0.71			
8	0.00417	1.2	61	0.50K	16.45	16.65		19.7
9	0.00417	1.2	·61	0.50K	16.85			
10	0.00625	1.2	104	0.25K	29.0			
11	0.00625	1.2	104	0.25K	27.2	28.1		26.7
12	0.00625	1.2	104	0.50K	30.0			
13	0.00625	1.2	104	0.50K	26.3			
14	0.00833	1.2	148	0.50K	32.4	34.7		32.4
15	0.00833	1.2	148	0.50K	37.0			
16	0.0125	1.2	235	0.25K	35.0			
17	0.0125	1.2	235	0.50K	41.6	40.0		39.0
18	0.0125	1.2	235	0.50K	43.4			
19	0.0250	1.2	497	0.25K	53.2			
20	0.0250	1.2	497	0.50K	50.0	50.9		47.9
21	0.0250	1.2	497	0.50K	49.4			
22	0.0500	1.2	1020	0.25K	56.4			
23	0.0500	1.2	1020	0.25K	52.4			
24	0.0500	1.2	1020	0.50K	57.2	54.8		54.4
25	0.0500	1.2	1020	0.50K	52.0			
26	0.0500	1.2	1020	0.50K	56.0			
27	0.0750	1.2	1540	0.50K	60.6	57.1		55.6
28	0.0750	1.2	1540	0.50K	53.6			
29	0.1000	1.2	2060	0.25K	62.0			
30	0.1000	1.2	2060	0.25K	55.2	57.5		56.7
31	0.1000	1.2	2060	0.50K	59.0			
32	0.1000	1.2	2060	0.50K	54.0			
33	0.1250	1.2	2590	0.50K	57.6	55.9		57.4
34	0.1250	1.2	2590	0.50K	54.2			

in the last column in Table III are the theoretical values of $\frac{g_K}{v}$ calculated by insertion in the equations derived later on.

Theoretical Remarks on the Calculation of the Affinity.

When the aqueous phase is not negligible compared with the lipid phase the following equation holds for the reaction between enzyme and triglyceride

$$v = v_1 + v_2$$

(v_1 = initial reaction velocity in the homogeneous phase, v_2 = initial reaction velocity in the lipid phase).

Let A_s be the initial surface per unit of volume of the mixture and m_E amount of enzyme adsorbed per surface unit of the lipid phase, then

$$v_2 = k_2 \times m_E \times A_s \qquad \text{(II)}$$

According to Freundlich's adsorption isotherm

$$m_E = k\ (E - m_E \times A_s)^{1/n} \qquad \text{(III)}$$

(k and n are constants, E = amount of enzyme per unit of volume of the mixture). Further

$$v_1 = k_1' \frac{(E - m_E \times A_s)\ a}{a + K_s} \qquad \text{(IV)}$$

(a = concentration of dissolved substrate, K_s = Michaelis' constant). As previously shown by the authors and confirmed in this work (IV) can be replaced with

$$v_1 = k_1 (E - m_E \times A_s)\ a \qquad \text{(V)}$$

When n is made equal to 1 in (III), which corresponds to a very large surface available for the amount of enzyme adsorbed, we get by insertion in (II)

$$v_2 = k_2 \frac{E \times A_s}{A_s + 1/k} \qquad \text{(VI)}$$

and equation V can be changed into

$$v_1 = \frac{1/k}{1/k + A_s} \times k_1 \times a \times E \qquad \text{(VII)}$$

(VI) is analogous to (IV), the surface per volume unit of the mixture, however, entering instead of substrate concentration and 1/k instead of K_s.

Calculation of the Affinity.

It is considered justified to make n = 1, as this assumption leads to equations which are satisfied by the experimental data. First v is corrected for v_1. The $v_1 = k_1 \times a \times E$ for the aqueous triglyceride phase in just saturated solution is calculated from the average value of $\frac{v_{uncorr}}{g_K \times m \times 10^5} = 0.558$ by multiplying by

1.2 (The concentration in the saturated solution is 1.2×10^{-5} m). For an arbitrary A_s-value

$$v_1 = 0.67 \times \frac{1/k}{1/k + A_s} \qquad \text{(VIIa)}$$

It is seen that v_1 decreases by increasing A_s. In Table III it is found that 1/k is about 100. If one calculates $v_2 = v - v_1$, the approximate correction for v_1 is $0.67 \times \frac{100}{100 + A_s}$.

The calculation of 1/k can be carried out graphically from equation VI transformed into (IX). For very great A_s-values (VI) can be written

$$v_{max} = k_2 \times E \qquad \text{(VIII)}$$

Hence

$$\frac{A_s}{v_2} = \frac{1}{v_{max}} (A_s + \frac{1}{k}) \qquad \text{(IX)}$$

This is the equation of a straight line with the ordinate $\frac{A_s}{v_2}$ and the abscissa A_s. In Fig. 2 is shown the graphical calculation of

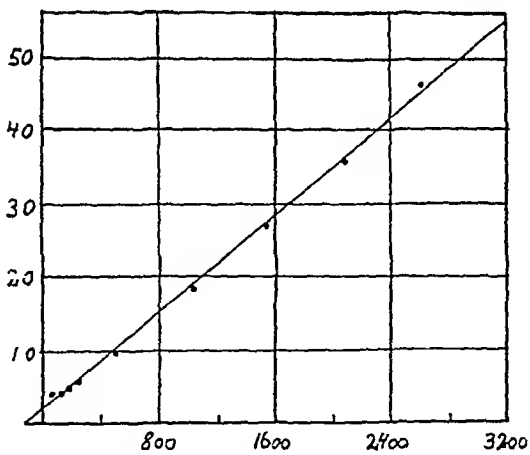


Fig. 2. Graphical determination of 1/k and v_{max} for the hydrolysis of tricaproin in heterogeneous mixtures. Abscissa A_s in cm²; ordinate $\frac{A_s}{v_2}$.

1/k and v_{max}. The distance from the point of intersection between the straight line and the abscissa axis to the zero point is 1/k = 130. v_{max} = 1/the slope of the straight line is equal to 60.2.

By inserting the values found for 1/k and v_{max} in (I, VIIa and IX) the figures in the last column of Table III are obtained.

For all the points — except the first one — a good agreement is found between observed and calculated values of v. The value found for $1/k = 130$ cm² per 250 cc system, 30° C and shaking with 250 double oscillations per min. indicates the substrate surface, where the reaction velocity has reached half its maximal velocity. This value is analogous to the MICHAELIS constant for homogeneous systems.

In homogeneous solution the affinity $1/K_s$ of pancreatic lipase to tricaproin was extremely low, that is K_s is very great compared with the substrate concentrations measured. When the system contains increasing amounts of lipid phase, a remarkable increase is demonstrated in the velocity of reaction, which rapidly reaches a maximal velocity by increasing substrate surface. k is a measure of the affinity between tricaproin and pig pancreas lipase in the surface layer betwen aqueous and lipid phase. As it was shown, 1/k is very small compared with the substrate surface measured, which means, that the affinity is very great.

A direct comparison of the affinities in the two phases is not possible, as their reciprocal values have the dimensions concentration and area of lipid phase per unit volume of reaction mixture respectively.

In our previous work on the affinity of pig pancreas lipase for some lower triglycerides in homogeneous solution it was shown, that the addition of $CaCl_2$ and sodium taurocholate did not influence the affinity between enzyme and substrate. Investigations on the possible influence of the above mentioned activators on the affinity in heterogeneous systems can not be carried out until sodium cholate is available.

Summary.

The affinity of pig pancreas lipase towards tricaproin in heterogeneous mixtures has been determined at 30° C in the presence of sodium taurocholate and calcium chloride with constant shaking, (250 double oscillations per min.), the initial velocity of the reaction of hydrolysis in the lipid phase being calculated in relation to the initial surface of the lipid phase. In the investigated tricaproin emulsions with increasing amounts of tricaproin a normal and approximately uniform frequency distribution was found with regard to the logarithm of the diameters of

the fatty particles. A conversion factor is computed from the relation between the total surface and the total volume of the particles and by means of this factor the total initial surface of the lipid phase in each enzymatic hydrolysis experiment can be calculated. — By the calculation of the affinity, FREUNDLICH's adsorption isotherm has been applied to the adsorption of the enzyme in the surface layer of the lipid phase. The reciprocal value of k, where k is measure of the affinity of lipase towards undissolved tricaproin, is under the given experimental conditions equal to 130 cm^2 per 250 cc reaction mixture.

This work was facilitated by a grant from "Christian X's Fond".

References.

SCHØNHEYDER, F., and K. VOLQVARTZ, Acta physiol. Scand. 1944, *7*, 376.

WEINSTEIN, S., and A. WYNNE, J. Biol. Chem. 1935—36, *112*, 641.

WILLSTÄTTER, R. and E. WALDSCHMIDT-LEITZ, Hoppe-Seyl. Z. 1923, *125*. 132.

From the Biochemical Institute, University of Stockholm, and the Chemical Laboratory, Karolinska Sjukhuset.

Determination of Blood Serum Citric Acid as Acetone.

By

PER W. KROG.

Received 29 September 1944.

Since NORDBÖ (1931) and SCHERSTÉN and collaborators (1930) detected citric acid as a constituent of the blood serum by different methods, this substance has attracted the attention of physiologists as well as practitioners. It has been proved that citric acid forms a link in the intermediate carbohydrate metabolism (MÅRTENSSON 1940 and bibliography there referred to). During pathological conditions the citric acid content of blood serum differs from its normal value. The normal content amounts to 17—27 γ per ml, the mean value being approximately 20. (NORDBÖ and SCHERSTÉN 1930, BENNI, SCHERSTÉN and ÖSTBERG 1930, MÅRTENSSON 1940, SJÖSTRÖM 1937, ÖSTBERG 1934, LINDQUIST 1936). An increased content of citric acid occurs during liver diseases, and with the aid of citric acid determinations it is sometimes possible to differentiate between jaundice due to damage to the liver tissue and that arising from obstruction of the bile duct (SJÖSTRÖM 1937).

At present the citric acid content of blood serum is determined by means of the THUNBERG method or the pentabromoacetone method.

The THUNBERG method (THUNBERG 1929) and modifications (ÖSTBERG 1931, 1934; GRÖNVALL 1938) employs the citric acid dehydrogenase and involves observation of the time necessary for the decolorization of methylene blue. Certainly this is not a strictly specific method. Other reducing substances also decolorize methylene blue. By using a dye with a lower redox potential,

e. g. indigo trisulphonate (GRÖNVALL 1938), it is possible to reduce the influence of those substances. Another source of error depends on the fact that a number of enzymes are involved in the reaction. At first the citric acid is converted into isocitric acid, and on this acid the dehydrogenase, actually an isocitric acid dehydrogenase (MARTIUS, 1938), will act. For this reason the presence of isocitric acid greatly shortens the decolorization time (EULER 1939). As isocitric acid also occurs in blood serum, it will interfere with the determination of the citric acid.

The pentabromoacetone method has been developed by PUCHER, SHERMAN and VICKERY (1936). In brief it consists in the oxidation of the citric acid by means of potassium permanganate in the presence of bromine. The pentabromoacetone produced is extracted from the oxidation mixture with petroleum ether and treated with aqueous sodium sulphide. A coloured substance is formed, and the colour is determined in a Pulfrich photometer.

For clinical purposes these methods are far from satisfactory. Too much time is required for the analyses. For this reason it seemed desirable to develop a more rapid method of citric acid determination. To some extent this is realized in the method described below. The greatest interest of this method, however, seems to lie in its apparently higher specificity. It gives lower values for the citric acid content of the blood serum. Added citric acid is, however, completely recovered.

The procedure consists in oxidation of approximately 20 γ citric acid by potassium permanganate. By this treatment the citric acid is converted into acetone. This is distilled and quantitatively determined. From the acetone value the amount of citric acid in the sample may be calculated. Previous investigators have used the same method for determining the citric acid contents of wine and milk, in which it occurs in great quantities (PRATT 1912, WILLAMAN 1916, KOGAN 1930, ROMANI 1931, EMILIANI 1933, BARTELS 1933, TÄUFEL 1933, BÄCKSTRÖM 1942). In blood serum, however, interfering substances, mainly proteins and acetone bodies, are present. These must be removed before the oxidation of the citric acid may take place.

Reagents.

1. Metaphosphoric acid, 5 per cent solution. Fit for use for a week if kept in a refrigerator.

2. Dilute sulphuric acid. 85 ml concentrated acid is diluted to 1 000 ml.
3. Buffer for neutralization. 381 g. $Na_3HPO_4 \cdot 12\ H_2O$ is dissolved in 4 000 ml water. 2 000 ml 3 N NaOH is added. A mixture of 50 ml buffer and 25 ml of the dilute sulphuric acid gives a pH value of approximately 2.
4. Potassium permanganate, N/5 000 solution, prepared by diluting N/10 $KMnO_4$.
5. KOH, exactly 14.5 N/solution.
6. Salicylaldehyde solution. To 10 g. Salicylaldehyde alcohol is added to 100 ml.

Note. To avoid errors due to acetone in the distilled water this must be boiled before use.

Procedure.

Precipitation of proteins. To 10 ml-metaphosphoric acid 3 ml of the blood serum is added. After standing for 10 minutes the sample is centrifuged. Approximately 11 ml limpid fluid is available.

Destruction of interfering substances. Acetone bodies, i. e. β-hydroxybutyric acid, acetylacetic acid and acetone itself, must be decomposed. For this purpose the solution is boiled with the dilute sulphuric acid mentioned above. By this treatment the β-hydroxybutyric acid is transformed into crotonic acid, whilst the acetylacetic acid forms acetone, carbon dioxide and traces of acetic acid. The acetone formed by the decomposition of the acetylacetic acid as well as the acetone preformed is boiled off in a 250 ml flask fitted with a ground-glass point. To 5 ml of the protein-free liquid 25 ml dilute sulphuric acid is added. To prevent bumping talcum is introduced. After boiling for 10 minutes 99.8 per cent of the hydroxybutyric acid and all the acetylacetic acid and acetone have disappeared. By this treatment the volume of the solution is reduced to 10—15 ml. In this solution the oxidation of the citric acid may take place.

Oxidation of the citric acid. On treating citric acid with potassium permanganate in acid solution acetone dicarboxylic acid is formed. When the acid solution of this substance is boiled it is decomposed into acetone and carbon dioxide. A detailed investigation of this reaction has been made by TÄUFEL and MAYR (1933).

In order to convert all the citric acid into acetone it is necessary to keep the pH of the solution at about 2. At a pH value below 1.9 the $KMnO_4$ is a too powerful oxidising agent; the citric acid is partly destroyed. At a pH higher than 2.3 the rate of decarboxylation of the acetone dicarboxylic acid is too slow, and a part of it will be destroyed. For this reason the solution must be buffered to a pH value of approximately 2.0. For this purpose 50 ml of the buffer mentioned above is added to the acid mixture in the 250 ml flask. To prevent loss of citric acid it is necessary to keep the concentration of $KMnO_4$ as low as possible. The $KMnO_4$ must be added in drops. In order to decompose the acetone dicarboxylic acid immediately after its formation the solution is kept boiling. The oxidation is carried out in the apparatus described below.

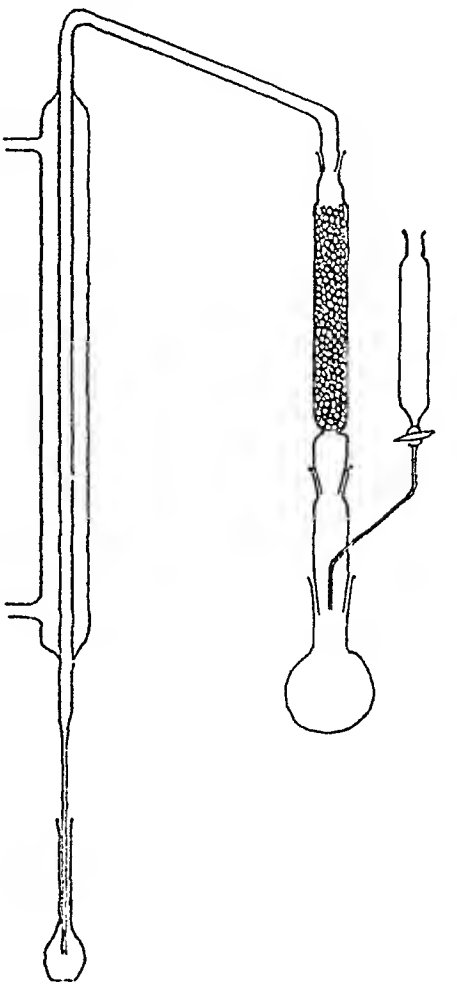


Fig. 1.

The solution containing citric acid is boiled in a 250 ml flask. To prevent bumping talcum is added. 100 ml N/5 000 $KMnO_4$ is placed in the funnel and added over half an hour to the boiling mixture. During this time the vapour containing acetone is condensed and retained in the glass tube, which is filled with glass beads. When all the $KMnO_4$ is consumed the heating is increased in order to distil off the acetone, which is collected in a 25 ml volumetric flask. When approximately 10 c.c. has been driven over (determined by comparison with another 25 ml flask containing exactly 10 ml fluid) the distillation is finished. In the distilled fluid the acetone is determined.

Determination of the acetone. As the citric acid content of the

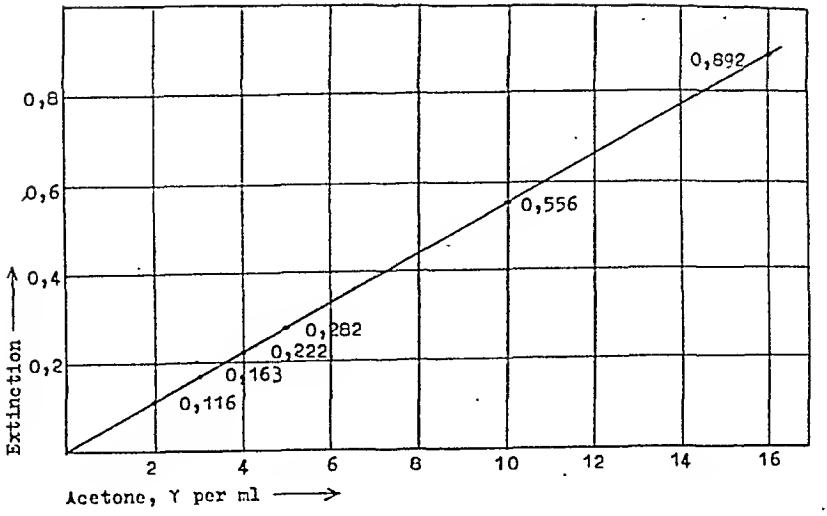


Fig. 2.

sample is very small, it is necessary to develop a highly sensitive and exact method for the determination of the acetone formed by the oxidation. The colorimetric method usually employed (URBACH 1931; NEUWEILER 1933) has proved to be efficient. It is based upon the condensation of acetone with salicylaldehyde in the presence of concentrated KOH. The product formed, disalicylalacetone, is an intensely red coloured substance. The colour is measured in a Pulfrich photometer. Some modifications of the present method have, however, proved necessary in order to increase the sensitivity. The following procedure is recommended:

To the acetone-containing fluid in the 25 ml volumetric flask 10 ml 14.5 N KOH and 5 ml salicylaldehyde solution are added. The mixture is made up to volume with water. In another flask a control test is prepared by using distilled water instead of the acetone-containing fluid. The flasks are heated on a water bath at 50° for 20 minutes. Afterwards they are allowed to cool at room temperature for half an hour. The extinction is finally determined in a Pulfrich photometer in cuvettes of 50 mm length and with filter S 53. The colour is very stable. Even after standing for 24 hours the extinction differs very little from the original value. In order to obtain exact values, however, the extinction should be measured within an hour after cooling the flasks. In order to calculate the acetone content an extinction curve must be constructed. An example is given below. The extinction values indi-

cated are not the calculated mean values, but the values estimated with the Pulfrich photometer. For each point only one analysis has been made. The values indicated below will therefore give a hint of the accuracy of the procedure for acetone determination.

It appears that the curve is a straight line. 10 γ acetone will give an extinction value of 0.558. From this it is calculated that an extinction value of 1.000 is given by 17.9 γ acetone.

Calculation of the citric acid content. On the basis of the quantity of blood serum employed, the molecular weights of acetone and citric acid and the extinction for pure acetone, the citric acid of the blood serum may be calculated according to the following formula:

$$E \cdot \frac{17.9 \cdot 13 \cdot 192}{3 \cdot 5 \cdot 58} = E \cdot 51 \ \gamma \text{ citric acid per ml blood serum.}$$

(E is the extinction read in the Pulfrich photometer).

Some values found by the method are given below.

Sample	Found by pentabromo-acetone method.	Found by "acetone" method.	Found by "acetone method" after addition of 20γ citric acid per ml.	Citric acid recovered, %.
1	11 γ/ml	7.2 γ/ml	26.8 γ/ml	98
2	19 »	13.4 »	33.5 »	100.5
3	23 »	14.1 »	33.6 »	97.5
4	18 »	12.8 »	31.5 »	93.5
5	40 »	28.1 »	48.0 »	99.5

It is seen that the values found by the "acetone" method are lower than those found by the pentabromoacetone method. Added citric acid is, however, almost completely recovered.

Summary.

The principle of the method is to convert the citric acid into acetone through oxidation by potassium permanganate, the acetone then being distilled and quantitatively determined.

Bibliography.

BARTELS, W., Z. Unters. Lebensm. 1933. *65*. 1.
BENNI, B., B. SCHERSTÉN and O. ÖSTBERG, Biochem. Z. 1930. *223*. 443.
BÄCKSTRÖM, H., Z. analyt. Chem. 1942. *123*. 107.
EMILIANI, F., Ann. chim. appl. 1933. *23*. 188.
EULER, H. v., and M. PLASS, Biochem. J. 1939. *33*. 1028.
GRÖNVALL, H., Fysiogr. Sällsk. Lund Förh. 1938. *7*. 40.
KOGAN, A. J., Z. analyt. Chem. 1930. *80*. 112.
LINDQUIST, N., Fysiogr. Sällsk. Lund Förh. 1936. *5*. 17.
MARTIUS, C., Hoppe-Seyl. Z. 1938. *257*. 29.
MÅRTENSSON, J., Nord. med. 1940. 253.
NEUWEILER, W., Klin. Wschr. 1933. I. 869.
NORDBÖ, R. und B. SCHERSTÉN, Skand Arch. Physiol. 1931. *63*. 124.
PRATT, D., U. S. D. A. Bur. of Chem. Circ. 1912. *88*. 1.
PUCHER, G. W., C. C. SHERMAN, and H. B. VICKERY, J. biol. chem. 1936. *113*. 235.
ROMANI, B., Ann. chim. appl. 1931. *21*. 496.
SJÖSTRÖM, P., Acta Chir. Scand. 1937. *79*. Suppl. 49.
THUNBERG, T., Biochem. Z. 1929. *206*. 109; Abderhaldens Handb. biol. Arbeitsmeth., Berlin und Wien 1933, Abt. IV, Teil I, 2017.
TÄUFEL, K. und F. MAYR, Z. analyt. Chem. 1933. *93*. 1.
URBACH, C., Biochem. Z. 1931. *236*. 164.
WILLAMAN, J. J., J. Amer. Chem. Soc. 1916. *38*. 2193.
ÖSTBERG, O., Skand. Arch. Physiol. 1931. *62*. 81.
ÖSTBERG, O., Z. ges. exp. Med. 1934. *94*. 442.
ÖSTBERG, O., Skand. Arch. Physiol. 1934. *67*. 265.

From the Laboratory for the Theory of Gymnastics and the Laboratory of Zoophysiology, University of Copenhagen.

Studies on the Initial Increase in O_2-capacity of the Blood at Low O_2-pressure.

By

ERLING ASMUSSEN and MARIUS NIELSEN.

Received 4 October 1944.

It is a well established fact, that the number of erythrocytes in the blood of individuals living at high altitude is higher than the number normal to individuals living at sea level. The change from sea level values to high altitude values has been followed — by means of haematocrit readings, determinations of O_2-capacity or haemoglobin concentration, or by blood counts — in several cases, where subjects moved from the lowlands to the mountains, as well as in low pressure chambers. When the ascent to high altitude — or the decrease in O_2-pressure — has been rapid, it is mostly found, that the change in O_2-capacity sets in early so that a considerable increase may be found already after a day or two in the height.

It has been well known to most investigators, that the observed higher haematocrit readings, blood counts etc. could be explained in two different ways, viz. either as due to an actual increase in the number of erythrocytes, or as the effect of a diminished plasma volume, and experimental data, supporting both points of view, have been collected. Nevertheless a certain confusion still seems to obscure the question, mostly because only a very few investigators realize that the blood changes during acclimatization to high altitude may follow in two stages, each needing its special explanation. SCHNEIDER (1921), who was well aware of this, in reviewing the literature on this problem, summa-

rizes that "the increase in hemoglobin observed, during short exposures to and during the early days at high altitudes, is largely or wholly due to a loss of fluid from the blood; while the permanent condition of acclimatization is the result of a new formation of red cells —". His conclusion is to a great part based on his own observations, including those from the Anglo-American expedition to Pikes Peak (1913). In the more recent reviews by PETERS and VAN SLYKE (1932) and by LUFT (1941) the increased O_2-capacity of the blood is explained as solely due to an actual increase in the number of erythrocytes, but no distinction between an initial and a later stage of the process is made. Experiments of SMITH, BELT ARNOLD and CARRIER (1924—25) are cited as giving evidence against the conception, that the plasma volume is diminished at high altitude, but as these authors present no experimental data covering the first 3—4 days of the sojourn at high altitude, no conclusions as to what happens with the blood during these first days can be based on their experiments.

ASMUSSEN and CONSOLAZIO (1940) found on Mt. Evans (4 300 m), that in two subjects the O_2-capacity in volumes per cent increased rapidly during the first two days, later at a more slow rate, and that the *total* O_2-capacity of the blood was practically constant or even slightly decreasing during the first 4—6 days. During this time the blood volume was markedly lower than at sea level, due to a considerable decrease of the plasma volume. Later the blood volume increased again, and the total O_2-capacity increased, indicating an augmented formation of red cells. These observations, therefore, give support to the conception formulated by SCHNEIDER. It was tentatively suggested that the decrease in blood volume during the first days of the sojourn at Mt. Evans was a regulation by means of which the organism could raise the percentage O_2-capacity of the blood until an actual increased erythropoiesis could be established. But it must be admitted, that the fall in blood volume might also be interpreted otherwise, e. g. as caused by a leaking-out of fluid from the capillaries, due to O_2-lack in the tissues.

It is the purpose of this paper under controllable conditions to repeat the experiments made on Mt. Evans, and to make an attempt to find out, whether the fluctuations in volume of cells and plasma in the blood are due to an actual excretion of water from the organism, or simply to a new distribution of the water inside the organism.

Methods etc.

All experiments were made in a low pressure chamber. In the control experiments the door of the chamber was kept open, so that normal atmospheric conditions prevailed; in the experiments simulating high altitude experiments, the pressure was lowered in the chamber to 450 or 435 mm Hg., corresponding to heights of about 4.5 km. Temperature, light etc. were the same in the controls and the low-pressure experiments. The chamber was well ventilated so that the composition of the air was as normal in-door air.

Blood samples, drawn without stasis from a cubital vein, were heparinizised, part of each sample centrifuged for half an hour at 3 000 rev/min in small glass tubes, part of it rotated in air at room temperature for 15 minutes in flasks of about 150 ml capacity and then analysed for O_2-capacity in the VAN SLYKE apparatus. On the small glass tubes, after centrifugation, the relative cell volume was measured, a drop of the plasma was then pipetted off and placed in the ZEISS Refractometer for determination of the plasma protein concentration. Blood volumes were determined by the CO-method as described by ASMUSSEN (1941).

Determinations of Cl^- in the urine were made according to VOLHARD —HARVEY (PETERS and VAN SLYKE (1932)). Na^+ and K^+ determinations were kindly made for us by Prof. A. KROGH. (Methods: see KROGH (1943) and HOLM-JENSEN (1944)).

The weight of the subjects was determined with an accuracy of 5 g by means of a special balance (KROGH and TROLLE (1936)).

Our subjects were young, healthy men, who previously had gone through a thorough medical examination and had satisfactorily passed an "aviators test" as worked out in the Zoophysiological Laboratory.

In our first experiment, the subject lived in the chamber at normal pressure for about 48 hours before the pressure was lowered (at $7^{a.\,m.}$) and kept at 450 mm Hg for about 60 hours. Determinations were made every day at $9^{a.\,m.}$ (fasting values) and at about $5^{p.\,m.}$ In subsequent experiments it was deemed necessary to avoid intake of food and drink. The subject therefore entered the chamber at $10^{p.\,m.}$, and slept in the chamber during the night. The experiment then started at about $9^{a.\,m.}$ next morning and was finished at $9^{a.\,m.}$ the following morning. The subjects were weighed before and after the experimental period, and the urine voided was collected and measured, in our latest experiments also analysed for Cl^-, Na^+ and K^+. In the short experiments there were no stools during the experimental period.

Determinations of O_2-intake, ventilation, cardiac output, pulse rate and blood pressure were also made. The results confirmed previous observations along the same line and will only be referred to occasionally.

Results.

Results from an experiment on A. H. (25 years, height 176 cm, weight 70 kgms) are presented in Fig. 1. It will be seen, that

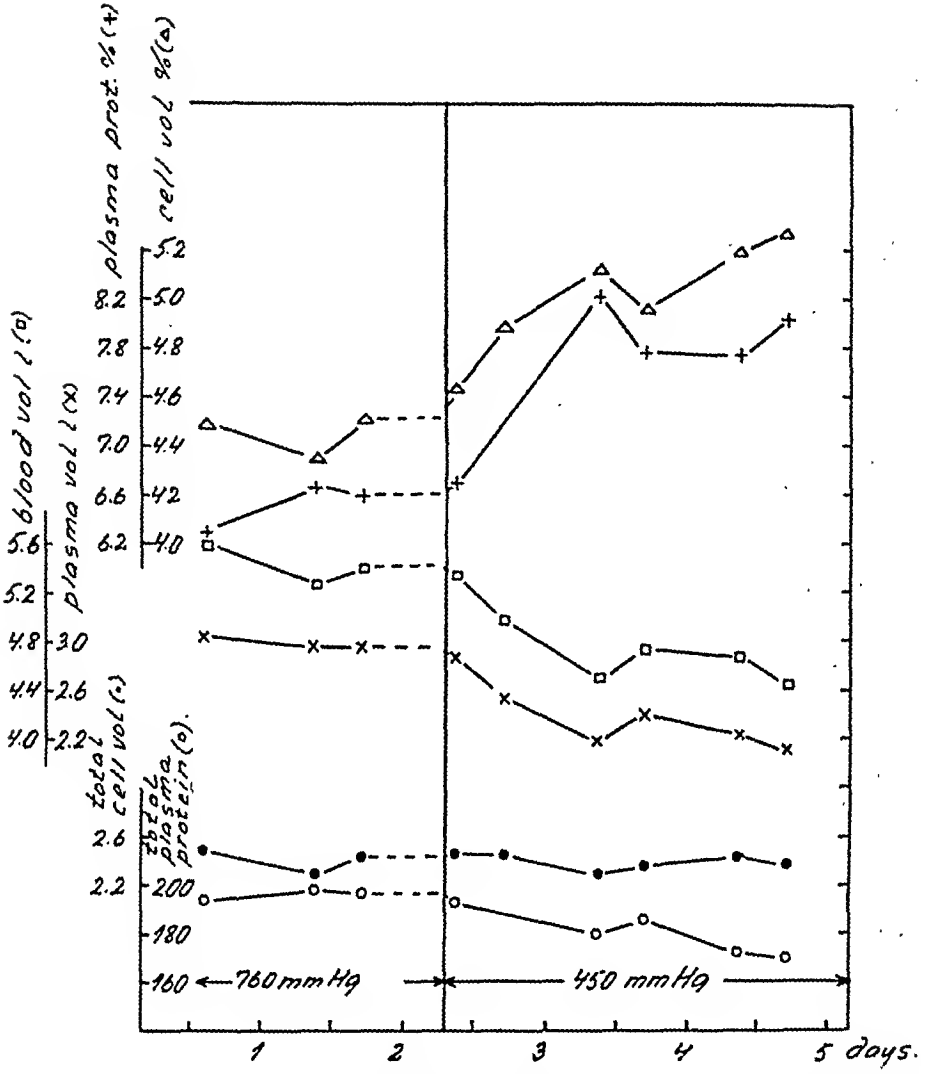


Fig. 1. Changes evoked in blood concentration and blood volume by lowering the atmospheric pressure from 760 mm Hg to 450 mm Hg. Subject A. H.

△ — △ Cell volumes %.
\+ — + plasma protein %.
□ — □ blood volume liters.
× — × plasma volume liters.
● — ● total cell volume liters.
○ — ○ total plasma protein grams.

the percentage cell volume (haematocrit-readings) and the plasma protein concentration undergo a marked increase, evident already a few hours after the pressure has been set at 450 mm Hg. Concomitant with this a corresponding decrease in the blood volume is observed. The curve representing the plasma volume and the almost horizontal line of the total cell volume show, that the decrease in blood volume is due to a loss of fluid from the plasma, and that the increased heamatocrit-readings (and the increased O_2-capacity in vol. % not presented in the fig.) are due to this

decrease of plasma volume, and not to an increased erythropoiesis. The increase in plasma proteins brings further support to this finding. This experiment confirms the results of SCHNEIDER (1921) and of ASMUSSEN and CONSOLAZIO (1940). The curve representing the total plasma protein is slightly decreasing, suggesting that the decrease in plasma volume is not solely due to a loss of water and salts, but that also some of the non-diffusable constituents of the plasma are lost.

From this experiment it was not possible to decide, whether the fluid loss from the blood was due to water excretion, or whether a new distribution of the water inside and outside the blood vessels had occurred. The subject had undergone a loss of body weight during the 2 days at low pressure (from 70.0 kgms to 67.7 kgms), but his appetite had been failing so that no decisive answer could be given to the question.

A second set of experiments (2 at normal pressure, and 2 at low pressure) was therefore performed on the same subject. During these experiments no food or drink was given, so that an accurate water balance could be made. The control experiments were made alternating with the low pressure experiments, and a week elapsed between each individual experiment.

The results from two such experiments, one control at normal atmospheric pressure, and one at a pressure of 450 mm Hg, are given in table 1.

The table shows, that fasting in itself produces an effect on the blood and the components of the blood similar to those of high altitude. But table 1 shows also, that the changes observed are much more pronounced at low barometric pressure than under normal conditions. The column headed "plasma proteins in blood" is calculated from the percentage plasma volume (100 — cell vol %) and the plasma protein % and shows the plasma protein content in per cent of whole blood. The percentage change in this is directly comparable with the percentage change in cell volumes per cent. At normal pressure they are of the same order of magnitude (+ 5, respectively + 6 %.), as should be expected. At low atmospheric pressure the cells are concentrated more than are the proteins (9 versus 6 %), indicating a loss of proteins from the blood (see later). The last four columns in table 1 show further that the loss of weight, fluid and chlorides is much greater at low barometric pressure than at normal pressure. From the loss of weight the total loss of water is estimated by subtracting 100 gms, which represent the approximate difference in weight

Table 1.

A. H.

Date (1943)	Blood vol l	Plasma vol l	Cell vol l	Cell vol %	Plasma prot %	Plasma prot. in blood %	Loss of Weight g	Total loss of Water g	Total Diuresis g	Total loss of Cl m. eq.
					normal atm. pressure.					
Oct. 13. 9a. m.	5.93	3.29	2.64	44.6	7.09	3.93				
Oct. 14. 9 a. m.	5.20	2.76	2.44	46.9	7.82	4.15				
increase (+) or decrease (—)	—0.73	—0.53	—0.20	+ 2.3	+ 0.73	+ 0.22	2,015	1,915	1,125	186.7
» » » in pCt.	—12 %	—16 %	—8 %	+ 5 %	+ 10 %	+ 6 %				
					low atm. pressure (450 mm Hg.)					
Oct. 20. 9a. m.	5.90	3.38	2.52	42.7	7.45	4.27				
Oct. 21. 9a. m.	4.90	2.62	2.28	46.6	8.49	4.53				
increase (+) or decrease (—)	—1.00	—0.76	—0.24	+ 3.9	+ 1.04	+ 0.26	3,590	3,490	2,015	293.5
» » » in pCt.	—17%	—22.5 %	—9.5 %	+ 9 %	+ 14 %	+ 6 %				

of the CO_2 expelled and the O_2 taken up (50 gms) during the 24 hours of the experiment, plus the weight of solids lost (salts, nitrogen etc., 50 gms). Of the total water lost a part is lost through the urine, the rest is evaporated from the lungs and the skin. The greater evaporation at low pressure is no doubt due to the increased ventilation and metabolism. But table 1 shows, that also the diuresis was largely increased at low pressure, and that a considerable amount of chlorides was excreted along with the water. Another set of experiments on the same subject gave corresponding results.

The amount of water lost greatly exceeds the volume of plasma lost, but naturally the loss of water (and chlorides) must be furnished by the whole phase of extracellular water (i. e. plasma, lymph etc.) and possibly also by the water inside the cells (intracellular water).

It is impossible from this experiment alone to decide which rôle these two compartments of body water play in the loss of water at high altitude, although the large amount of chlorides excreted indicates, that the extracellular water plays a dominant rôle. In order to make a further approach to the solution of this question a new set of experiments was made, in which the total excretion of sodium and potassium was determined during a 24 hour period, at normal atmospheric pressure, and at a pressure of 435 mm Hg. The subjects (L. 21 years, height 184 cm, weight 72 kgm, and K. 23 years, height 181 cm, weight 74 kgm) received no food or water in the experimental period and 15 hours previously.

According to GAMBLE, ROSS and TISDALL (1923) and PETERS and VAN SLYKE (1932) the loss of extracellular water can be estimated from the formula

$$\frac{Na - 0.425\ K}{148} = \text{liters of extracellular water lost}$$

where Na and K represent milligram equivalents of Na and K excreted in excess of intake (in our case, therefore, the total excretion), the factor 0.425 expresses the proportion of $\frac{Na}{K}$ in muscles and 148 is the average milliequivalents of Na found in extracellular fluids. Similarly the loss of intracellular water can be estimated from the formula

$$\frac{K - 0.017\ Na}{112} = \text{liters of intracellular water lost,}$$

where 0.017 is the proportion of $\frac{K}{Na}$ in extracellular fluids and 112 is the average content of K (in milliequivalents) of cells (muscle tissue).

Table 2 contains the experimental data from such experiments on the subjects L and K. It will be noticed, that K reacts with changes in blood concentration (cells and plasma proteins) in the same way as A. H. at low barometric pressure. He also looses more weight than under normal pressure and his excretion of water and salts is greatly increased at high altitude. L, whose blood undergoes a much smaller concentration than does K's, although he increases his percentage red cell volume considerably more at low pressure than at normal atmospheric pressure, does not loose much more water or excrete more urine and salts at 435 mm Hg. than at 760 mm Hg.

From the data of table 2 and by means of the formulae given above, the part of the water loss played by the various compartments of body water is estimated, and a balance sheet is drawn as shown in table 3. The table shows, that under normal barometric conditions the water loss is mainly derived from the extracellular water, nothing from the intracellular phase. The rest, 530 respectively, 430 cc may conveniently be explained as oxidation water and as water made free by depletion of the carbohydrate stores ("glycogen factor" of GAMBLE, ROSS and TISDALL.) At low pressure this last component is greatly increased in both subjects, and we are not in position to determine the source of this water. Of direct interest to the present investigation is the fact, that the amount of extracellular water lost is greatly increased in K (in whom also a small amount of intracellular water is lost) whereas in L no more extracellular water is lost at low pressure than at normal barometric pressure.

If the loss of extracellular water alone should explain the concentration of the blood, then one would expect to find that the percentage decrease in extracellular fluid (including the blood plasma) were of the same order of magnitude as the percentage increase in non-diffusable plasma components, (e. g. the plasma proteins). Further, from the percentage decrease in extracellular fluid (and hence plasma) one can estimate the percentage decrease in blood volume, and from this again the increase in cell volumes % to be expected under the assumption of a constant total cell volume. (The experiments on A. H. showed that the

Table 2.

Subj.	Date (1944)	Cell vol %	Plasma prot %	Cell vol increase %	Plasma-prot. increase %	Plasma-prot. increase in blood %	Loss of Weight g	Total loss of Water g	Diuresis g	Total loss of Na^+ m. eq.	Total loss of K^+ m. eq.	Total loss of Cl^- m. eq.
		normal atm pressure.										
L.	May 25. 9a. m.	42.5	7.09									
	May 26. 9a. m.	44.0	7.72	3.5 %	8.5 %	5.9 %	1,840	1,740	665	180.9	3.6	125.2
K.	May 25. 9a. m.	42.6	7.29									
	May 26. 9a. m.	45.0	8.02	5.6 %	10.0 %	5.7 %	1,420	1,320	445	131,8	2.4	105.6
		low pressure (445 mm Hg)										
L.	June 8. 9a. m.	44.7	7.20									
	June 9. 9a. m.	48.2	8.02	7.8 %	11.4 %	4.5 %	2,090	1,990	525	169.3	2.6	83.3
K.	June 8. 9a. m.	43.2	7.22									
	June 9. 9a. m.	49.3	8.13	14.1 %	12.6 %	0.5 %	2,540	2,440	1,345	212.4	8.6	153.6

cell volume actually decreased, by 9 % in his case, due to the fasting).

If we estimate the total amount of extracellular fluid to 25 % of the body weight, we get the following calculated losses of extracellular water (table 4) to compare with the concentrations of the blood actually found (comp. table 2).

It will be seen, that with exception of the experiment at normal pressure on L, the found concentrations are two to three times greater than the concentrations calculated under the assumption, that the loss of extracellular fluid was the only case of the concentration of the blood. (The difference between found and calculated values at low pressure is smaller for the plasma proteins than for the cell volumes, presumably due to a loss of protein from the plasma (see below).

The only plausible explanation seems to be, that, besides the loss of extracellular fluid, a loss of water from the blood plasma to the extravascular fluid (or to the tissue cells) must have occurred. Such a loss may be due to an increased permeability of the capillaries caused by the oxygen deficiency in the tissues, and to a higher capillary blood pressure, due to a dilatation of the arterioles and capillaries. An increased permeability of the capillary walls at low O_2-pressure, even allowing proteins to escape from the blood, seems to be indicated by the relatively slight increase in plasma proteins, compared with the actual decrease in plasma volume found on A. H. and presented in table 1 as well as in the observation, that the total amount of plasma proteins in fig. 1 shows a decrease at low O_2-pressure. Also the fact, that the proteins calculated as pCt of the whole blood (table 1 and 2) shows a much smaller concentration than does the cell volume, confirms this assumption.

Conclusions and discussion.

The conclusions to be drawn from our experiments, are: The increase in O_2-capacity, cell volume etc. which is observed in the blood during the first days at high altitude — or low O_2-pressure — is due to a concentration of the blood. There is no increase in the total amount of red cells during this time. Also during a 40 hours' period of fasting a concentration of the blood occurs. but the concentration during a fasting period at *low* pressure is considerably greater than the concentration during a fasting pe-

Table 3.

L

normal atm. press.				*low atm. press.* (135 *mm H*			
loss of weight	1,840 gms			2,090 gms			
solids + (CO_2—O_2)	100 »			100 »			
total loss of water	1,740 »	extra cell.	1,210 gms	1,990 »	extra cell.	1,13	
		intra cell.	0 »		intra cell.		
		water of			water of		
urine	665 »	oxyd. etc.	530 »	525 »	oxyd. etc.	8 6	
evaporated water	1,075 »			1,465 »			

K

normal atm. press.				*low atm. press* (135 *mm H*			
loss of weight	1,420 gms			2,540 gms			
solids + (CO —O)	100 »			100 »			
total loss of water	1,320 »	extra cell.	890 gms	2,440 »	extra cell.	1,41(	
		intra cell.	0 »		intra cell.	5(	
		water of			water of		
urine	445 »	oxyd. etc.	430 »	1,345 »	oxyd. etc.	98(	
evaporated water	875 »			1,095 »			

riod at *normal* pressure. This concentration is accompanied by a loss of water from the organism. The main part of this water derives from the extracellular fluid of the body including the plasma and can explain one third to one half of the observed blood concentrations (table 4). (In the experiment at normal pressure on L. it explains the whole blood concentration). The other part of the blood concentration must be due to an altered distribution of water between the blood and the rest of the body.

The loss of water from the blood at *low* pressure in excess of the loss of water from the blood during fasting at *normal* pressure represents the effect of the low O_2-pressure on the blood components. In K. this excess loss is due both to an increased excretion of water and to an increased movement of water from the blood out into the extravascular fluids. This latter is due, probably to an increased filtration of water through the capillary walls, caused by an increased permeability of the capillaries and an increased capillary blood pressure. The dilatation of arterioles and capillaries at the increased rate of circulation no doubt will increase the capillary blood pressure. We are unable to explain, why in K. also during fasting at *normal* pressure a consider-

able amount of water was lost from the blood to the tissues (as indicated by the great difference between concentrations calculated and found in table 4). In this case the circulation was quite normal.

Table 4.

		calculated loss in extra cell water	found increase in plasma prot. %	calculated loss in blood vol.	calculated increase in cell vol. %	found increase in cell vol. %
normal	L.....	6.7 %	8.5 %	3.8 %	4.0 %	3.5 %
	K.....	4.8 %	10.0 %	2.8 %	3.1 %	5.6 %
low press.	L.....	6.3 %	11.4 %	3.5 %	3.6 %	7.8 %
	K.....	7.6 %	12.6 %	4.3 %	4.6 %	14.1 %

In L. at low pressure the excretion of water was not increased beyond the values found during fasting at normal pressure. The observed greater concentration of his blood at low pressure therefore must be due solely to an escape of water from his blood, and also in his case the most probable explanation seems to be, that the filtration has increased.

As mentioned above both K. and A. H. showed an excess loss of water from the body at low O_2-pressure. This loss was partly due to an increased evaporation, partly — and mainly — to an increased diuresis. Whereas the increased evaporation may be explained by the increased ventilation and metabolism at low O_2-pressure, the cause of the increased diuresis is not easily understood. BIRLEY (cited from SCHNEIDER 1921) is, as far as we are aware, the first who noticed an increased diuresis at low O_2-pressure (on aviators), and he even suggested, that the increase in O_2-capacity at low O_2-pressure was chiefly due to this. Our experiments have shown, that only part of the concentration of the blood can be due to the loss of water from the body, and even that not in all subjects. Whether the increase in diuresis at low O_2-pressure, therefore, may be looked upon as being due to a special mechanism, the purpose of which is to increase the O_2-capacity of the blood, or whether it is the result of some other unknown phenomenon, incidentally increasing the concentration of the blood, still seems to be an open question.

Summary.

Determinations of red cell volumes, plasma proteins, blood volumes and water balanee were made in a low pressure chamber at 435—450 mm Hg barometrie pressure. It was found that the increase in cell volume — and O_2-capacity — during the first days at low pressure was due to a diminished plasma volume, and paralleled by an increase in plasma protein concentration. A similar concentration of the blood was found during fasting (40 hours) at normal barometric pressure, but a much greater coneentration of the blood was observed when the subjects fasted at low barometric pressure. In two of the three subjects the increased eoneentration of the blood at low pressure was accompanied by an extra loss of water from the body. From an estimation of the part played by the extracellular water in this loss, it was found, that about one half of the concentration of the blood was caused by loss of extraeellular body water. The other part of the eoncentration of the blood — and, in the third subject, the whole extra eoneentration at low pressure — was due to a leaking-out of fluid from the capillaries, presumably eaused by the inereased eapillary blood pressure and an increased permeability of the eapillary walls at low O_2-pressure. While the excess filtration out of the blood no doubt is a sign of insuffieieney of the capillaries, incidentally increasing the oxygen eapaeity of the blood, it is difficult to decide whether the excess excretion of water (increased diuresis) is the result of a regulation, serving the oxygen supply, or whether it is due to other causes.

References.

Asmussen, E. and F. Consolazio, Amer. J. Physiol. 1941, *132*. 555.
Asmussen, E., Acta physiol. scand. 1942, *3*. 156.
Gamble, J. L., G. S. Ross, and F. F. Tisdall, J. biol. Chem. 1923. *57*. 633.
Holm-Jensen, Ib., Kgl. Fysiogr. Sälsk. Handl. 1944. N. F. *55*. Nr 5.
Krogh, A. and C. Trolle, Skand. Arch. Physiol. 1936. *73*. 159.
Krogh, A., Acta physiol. scand. 1943. *6*. 203.
Luft, U. C., Ergebn. Physiol. 1941. *44*. 256.
Peters, J. P. and D. D. Van Slyke, Quantitative clinical chemistry I—II. Baltimore 1932.
Schneider, E. C., Physiol. Rev. 1921. *1*. 631.

From the Department of Physiology, University of Lund.

The Analgetic Action of Morphine, Eserine and Prostigmine Studied by a Modified Hardy-Wolff-Goodell Method.

By

SIGVARD FLODMARK and THURE WRAMNER.

Received 4 October 1944.

From experiments on cats SLAUGHTER and MUNSELL (1940) claim that prostigmine potentiates the analgetic effect of morphine. In confirmation of this SLAUGHTER, PARSONS and MUNAL (1940) report from investigation on patients suffering from different painful diseases that the patients experienced as excellent a relief from pain after 8 mg morphine in combination with 0.5 mg prostigmine as after 15 mg morphine alone. Using the HARDY-WOLFF-GOODELL method of measuring pain thresholds in man ANDREWS (1942), however, concludes that prostigmine does not significantly potentiate the analgetic effect of morphine. SLAUGHTER and GROSS (1940) observed that eserine potentiated the tonic action of morphine on the intestines of the dogs and the depressor effect on the blood pressure of the cats. Subcutaneous administration of 5 mg morphine per kg body-weight in dogs causes an inhibition of the serum choline esterase (SLAUGHTER and LACKEY, 1940). From their findings SLAUGHTER and coworkers concluded that morphine in some way or other acts through a cholinergic mechanism.

The possibility of a humoral mediator in the central nervous system has been intensively discussed recently. Some of the earlier investigators suggest the possibility that acetylcholine may normally be liberated in some synapses in the brain. HENDERSON and WILSON (1936) found that eserine injected in small quanti-

ties into the human cerebral ventricles produced a reaction in all respects very closely resembling that to acetylcholine. FELDBERG and SCHRIEVER (1936) observed that acetylcholine appeared in the cerebrospinal fluid of dogs after the intravenous injection of eserine. In experiments on cats EMMELIN and JACOBSOHN (1944) report that eserine and prostigmine injected intrathecally has an identical depressant effect on respiration and intestinal motility.

By stimulating the cerebral motor cortex in cats and studying the muscular responses after intracarotid injection of acetylcholine and eserine MCKAIL, OBRADOR and WILSON (1941) failed to obtain conclusive information regarding the possible action of acetylcholine as a quick transmitter in cerebral synapses. They found that small quantities of eserine usually depressed the responses to stimulation of the cortex. SCHWEITZER and WRIGHT (1937) observed on cats under chloralose that eserine augmented spinal reflex responses but that prostigmine depressed them. BÜLBRING and BURN (1941) found on dogs no difference in the action of eserine and prostigmine on the kneejerk. Both were depressant. On patients with evidence of pyramidal-tract involvements KREMER (1942) observed that prostigmine and acetylcholine, injected intrathecally, depressed muscle tone and spinal reflexes. No changes in the sensation were observed. Eserine, however, after initial motor depression produced increased muscle tone and spinal reflexes and caused striking sensory changes, including facilitation of sensory transmission.

With the elaborating of the method of HARDY, WOLFF and GOODELL (1940) a reliable method for determining pain thresholds on man became available. In view of the conflicting reports on the central effects of eserine and prostigmine, and the lack of correspondence between earlier experiments into the interaction between the analgetic effect of morphine and prostigmine on animals and man, it seemed of interest to us to reinvestigate these problems.

Method.

The method used involves a minor modification of that of HARDY, WOLFF and GOODELL (1940). Radiant heat of known and easily variable intensity was employed to cause pain. Fig. 1 demonstrates the apparatus.

The light from a 500 watt lamp is focussed by a condensing lens through a filter, containing the dyes fuchsin and tartrasine, and through a camera shutter on to the forehead of the subject. The intensity of the light is controlled by means of a rheostat in series with the lamp. The light is allowed to act on the subject for 3 sec., the time being controlled by a chronometer. The pain threshold was assumed to be reached when the subject felt a distinct sharp stab of pain at the end of this time. The intensity of the radiation is measured in g.cal./sec/cm² by a thermal couple made of copper and constantan. To the one junction is fastened a blackened silver-plate with the same diameter as

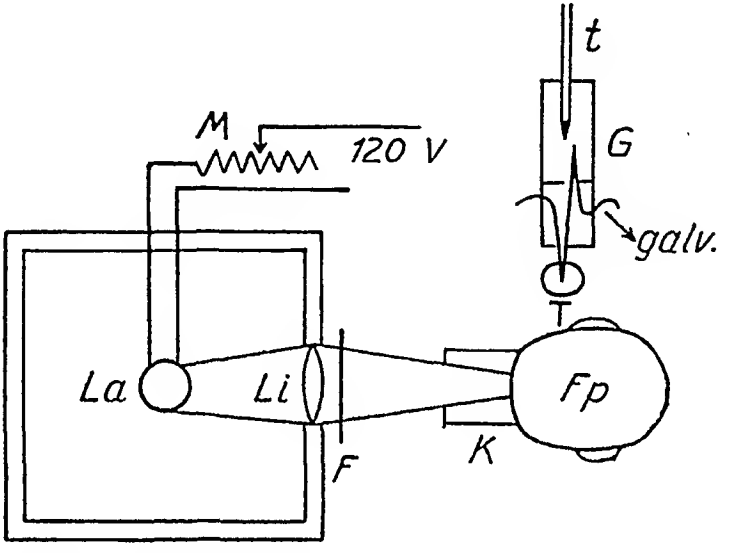


Fig. 1. Apparatus for determining pain thresholds in man.

Fp.: The subject. *K.:* Camera with shutter.
F.: Colour filter. *Li.:* Condensing lens.
La.: 500 watt lamp. *M.:* Rheostat.
T.: Thermal couple with silver-plate.
G.: Glass-reservoir. *t.:* Thermometer.

the camera shutter. The second junction is kept in a water bath at a constant temperature. The thermal current is measured by a specular galvanometer calibrated empirically.

Our method differs from that used by HARDY, WOLFF and GOODELL in one point. We use a colour filter and do not blacken the forehead of the subject with Indian ink.

To provoke pain three times more radiant energy is required when using radiant heat from a light source than when using heat rays alone (SONNE, 1921). The greater the wave lengths of the rays the more they act upon the pain receptors. According to SONNE this is due to the fact that the greater the wave lengths of the rays the more these are absorbed into the superficial layers of the skin, where the receptor organs for pain are situated. The same opinion is held by HARDY and MUSCHENHEIM (1936).

In order to ensure total absorption of the radiation and to eliminate possible effects due to the penetration of the rays below the skin surface, HARDY and co-workers blackened the forehead of the subjects with Indian ink. In our opinion this procedure is not very advisable. It is difficult to obtain the same thickness in the layer of Indian ink at every test, and in consequence it becomes difficult to obtain comparable values of the pain thresholds. When washing off the Indian ink superficial injuries to the skin can hardly be avoided. This fact may probably change the irritability of the skin. Further, the layer of Indian ink as such seems to us to change the subjective sensation of pain in an undefined manner.

In order to get heat rays which are absorbed into the superficial layers of the skin as much as possible we use the colour filter mentioned above. In this way, as controlled by spectroscopic examination, almost all rays with wave lengths shorter than 5900 Å are eliminated.

An obvious disadvantage in our method is the fact that the rays reflect in some degree from the unblackened skin of the forehead. According to SONNE (1921) this reflection is very constant, however, and is independent of the wave lengths. The reflection always amounts to 35 %.

Our experiments were performed on 19 students of both sexes, in good health and aged between 20 and 24. Before the tests, the subjects were acclimatized in the room for half an hour. The examined substances were administrated subcutaneously. The substances were not injected until two successive determinations of pain thresholds gave the same values. None of the subjects knew the nature of the injected substances. After the injection we determined the pain threshold every five minutes until the analgetic effect was observed, and then every ten minutes until the maximal effect was reached. After that the pain thresholds were determined only every 15 minutes. On four of the subjects we made blind tests, in which physiological NaCl-solution was injected. In these experiments we determined the pain threshold every ten minutes during two hours. The maximal deviation from the original value was $\pm$ 1.1 %, which therefore may be considered as the normal variations of the method.

Results.

The arithmetic mean of the normal pain thresholds of all our 19 subjects was 0,209 g. cal./sec./cm^2.

To compare the analgetic effect of 15 mg morphine hydrochloride alone with that of a combination of 8 mg morphine hydrochloride and 0.5 mg prostigmine[1] we used five subjects and found that morphine raised the pain threshold about 30 %,

[1] We used a brand of prostigmine, "neostigmin", manufactured by A. B. Leo, Hälsingborg, to which we wish to express our gratitude for generous supplies.

while the combination raised it about 40 % as shown in fig. 2. Between the two injections there was an interval of one week.

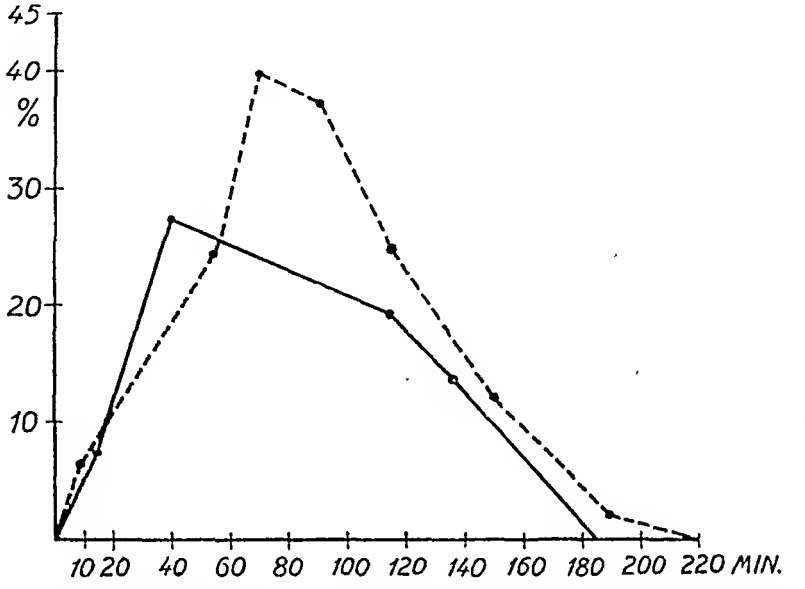


Fig. 2. Arithmetic mean of pain threshold raising effect of 15 mg morphine (————) and of 8 mg morphine+0.5 mg prostigmine on five subjects (– – – –).

The effect of 1 mg prostigmine on the pain threshold appears from table 1.

Table 1.

Subj.	Time until the effect occurs	Time until max. effect	Duration of the effect	Maximal effect on the pain threshold
1.	10 min.	35 min.	75 min.	+23.5 %
2.	35 »	35 »	70 »	+20.0 %
3.	20 »	30 »	65 »	+17.7 %
4.	25 »	45 »	55 »	+15.2 %
5.	15 »	40 »	90 »	+20.3 %
6.	40 »	55 »	70 »	+22.1 %
7.	25 »	50 »	85 »	+22.0 %
8.	30 »	40 »	100 »	+22.1 %
9.	10 »	40 »	85 »	+22.0 %

To investigate whether prostigmine potentiates the analgetic effect of morphine hydrochloride we used ten subjects. One of them did not react to prostigmine, nor did prostigmine on this subject potentiate the analgetic effect of morphine hydrochloride. In the remaining subjects, however, prostigmine clearly

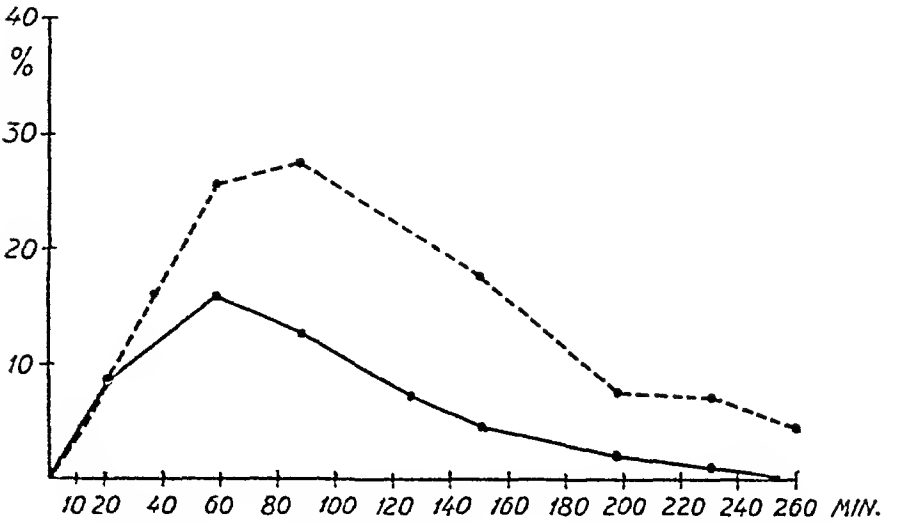


Fig. 3. Arithmetic mean of pain threshold raising effect of 8 mg morphine and 0.5 mg prostigmine simultaneously injected into ten subjects (– – – –); total of the arithmetic means of the pain threshold raising effect of 8 mg morphine and of 0.5 mg prostigmine injected separately into the same subjects one week later (————).

potentiated the action of morphine. The arithmetic mean of all the experiments shows that the maximal analgetic effect of 8 mg morphine hydrochloride and 0.5 mg prostigmine injected simultaneously is almost twice as great as the summated maximal effects of the two substances injected separately. The duration of the morphine action was prolonged as demonstrated in fig. 3.

More detailed figures from three subjects of table 1 are given in fig. 4.

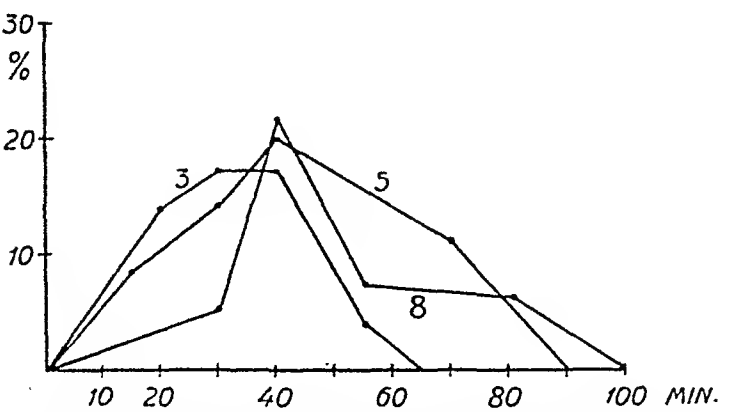


Fig. 4. Pain threshold raising effect of 1 mg prostigmine. From a series of nine subjects three representative subjects are selected.

The influence of eserine salicylate on the pain threshold was studied on four subjects. From fig. 5 it will be seen that 1 mg eserine salicylate raises the pain threshold roughly about 30 %.

We have not examined the effect of eserine on the analgetic action of morphine because of the subjective malaise, which was caused by eserine.

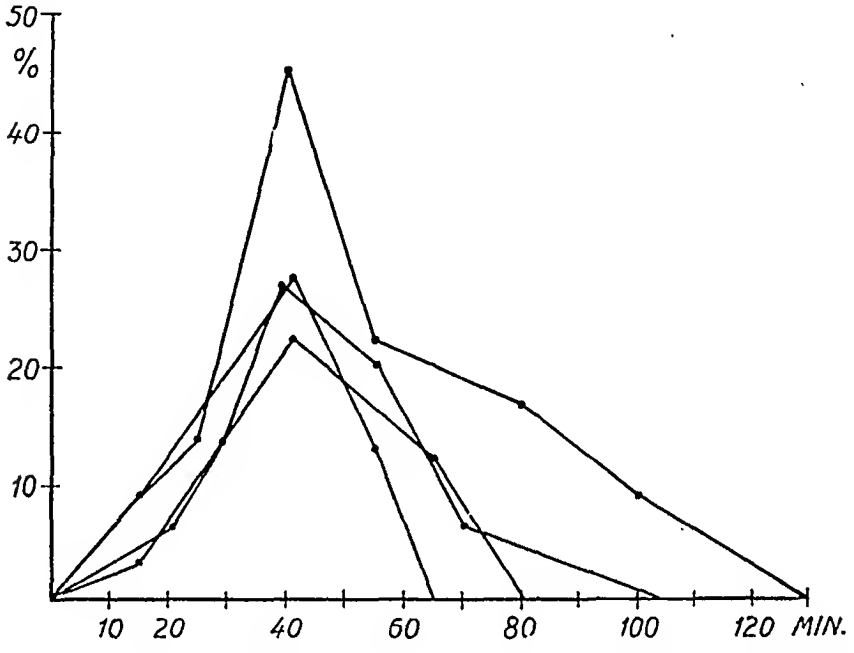


Fig. 5. Pain threshold raising effect of 1 mg eserine on four subjects.

Discussion.

We have found that prostigmine potentiates the analgetic effect of morphine hydrochloride. Both the maximal effect and the duration were increased. This fact is in conformity with the findings of SLAUGHTER and co-workers, where the subjective relief from pain in clinical cases was used as an indicator. ANDREWS, however, when using the original method of HARDY, WOLFF and GOODELL, found no significant potentiation either on normal persons (2 in number) or on addicts to morphine (15 in number). To this investigation objections may, however, be raised. The number of normal persons investigated is too small to permit of generalized conclusions. Among our ten subjects there was one who showed no potentiation. Further, it seems to us inadequate to use addictors who, according to ANDREWS' observations,

show abnormally small responses to morphine (20 mg morphine raised the pain threshold only about 5 %). Although the change in the pain threshold is abnormally small it is evident from the diagrams of ANDREWS that the analgetic effect of morphine is raised to double the original level by prostigmine. (20 mg morphine + 1 mg prostigmine raised the pain threshold about 10 %). In our opinion there is a clear potentiation in ANDREWS' experiments, also.

In our experiments both eserine and prostigmine raise the pain threshold. This effect may to some extent be due to an increased blood flow through the skin, as observed by PERLOW (1939). Besides, a central point of attack seems likely. In experiments on animals several investigators found that eserine and prostigmine are closely related, as judged by their action on the central nervous system. (MCKAIL, OBRADOR and WILSON 1941; SCHWEITZER and WRIGHT, 1937; BÜLBRING and BURN 1941; EMMELIN and JACOBSOHN, 1944). In man KREMER (1941) observed in agreement with the findings of these authors in animals that the dominant action of prostigmine on the C. N. S. is depressant. Studying eserine, he found in conformity with HENDERSON and WILSON (1936), who also experimented on man, that the central action of this drug is mainly stimulant. All observers agree in assuming a central action of the two substances, but disagree in estimating how much of the central action is due to a vascular and how much to a neurogenic component. MCKAIL, OBRADOR and WILSON (1941) studied the effects on the excitability and transmission of impulses in central nervous system of different drugs acting upon the cerebral circulation. They found that carbon dioxide, adrenaline and cerebral ischaemia produced the same depressant action on the cortical responses as acetylcholine, eserine and prostigmine. Whatever the fundamental mechanism, the action of acetylcholine, prostigmine and eserine is certainly central. Obviously too little is known about the point of attack and mode of action of eserine and prostigmine in the C. N. S. to explain why prostigmine potentiates the analgetic action of morphine. The suggestion of SLAUGHETR et al. (1940) that morphine in some way or other acts through a cholinergic mechanism is in good agreement with the observation presented in this paper.

Summary.

1. A slight modification of the Hardy-Wolff-Goodell method for measuring pain threshold on man is described.

2. Eserine raises the pain threshold.

3. Prostigmine raises the pain threshold.

4. Prostigmine greatly potentiates and prolongs the analgetic action of morphine.

References.

ANDREWS, H. L., J. Amer. med. Ass. 1940. *120*. 525.
BÜLBRING, E., and J. H. BURN, J. Physiol. 1941, *100*. 337.
EMMELIN, N., and D. JACOBSOHN, Acta Physiol. Scand. in print.
FELDBERG, W., and H. SCHRIEVER, J. Physiol. 1936, *86*. 277.
HARDY, J. D., H. G. WOLFF, and H. GOODELL, J. Clin. Invest. 1940. *19*. 649.
HARDY, J. D., H. G. WOLFF, and H. GOODELL., Ibidem 1940. *19*. 659.
HARDY, J. D., H. G. WOLFF, and H. GOODELL, Ibidem 1940. *20*. 63.
HARDY, J. D., Ibidem 1934. *13*. 615.
HARDY, J. D., and C. MUSCHENHEIM, Ibidem 1936. *15*. 1.
HENDERSON, W. R., and W. C. WILSON, Quart. J. Exp. Physiol. 1936. *26*. 83.
KREMER, M., Ibidem 1942. *31*. 337.
MCKAIL, R. A., S. OBRADOR, and W. C. WILSON, J. Physiol. 1941. *99*. 312.
PERLOW, A., J. Pharmacol. 1939. *66*. 66.
SCHUMACHER, G. A., H. GOODELL, J. D. HARDY and H. G. WOLFF, Science 1940. *92*. 110.
SCHWEITZER, A., and S. WRIGHT, J. Physiol. 1937, *88*. 459, 476.
SCHWEITZER, A., and S. WRIGHT, Ibidem 1937, *89*, 165. 384.
SLAUGHTER, D., and E. G. GROSS, J. Pharmacol. 1940. *68*. 96.
SLAUGHTER, D., and D. W. MUNSELL, Ibidem 1940. *68*. 104.
SLAUGHTER, D., and R. W. LACKEY, Proc. Soc. exp. Biol. n. y. 1940. *45*. 8.
SLAUGHTER, D., J. C. PARSONS and H. D. MUNAL, J. Amer. med. Ass. 1940. *115*. 2058.
SONNE, C., Acta Med. Scand., 1921. *54*. 336.

From the Department of Physiology, University of Lund.

Some Effects of Acetylcholine, Eserine and Prostigmine when Injected into the Hypothalamus.

By

NILS EMMELIN and DORA JACOBSOHN.

Received 6 October 1944.

The conception that acetylcholine is in some way engaged in the process underlying the excitation of central nerve cells is supported by recent experiments. Acetylcholine has been extracted from the brain (for references see MACINTOSH 1941), and nervous tissue is capable of synthetizing acetylcholine (MANN et al. 1938). Observations on the action of acetylcholine, eserine and prostigmine on cerebral and spinal functions suggest that acetylcholine may act as a central transmitter; of special interest is the observation that eserine potentiates the central action of acetylcholine (see BÜLBRING and BURN 1941). It is also claimed that acetylcholine is liberated on the stimulation of afferent nerves (see BÜLBRING and BURN 1941). If KCl is injected into the perfused brain of the cat the concentration of acetylcholine in the perfusate is increased (CHUTE et al. 1940). Acetylcholine can readily be removed from brain cells, since the grey matter is very rich in acetylcholine esterase (NACHMANSOHN 1939).

In this connection reference is frequently made to experiments in which drugs acting on the autonomous nervous system were injected into the ventricles of the brain. Pilocarpine injected into the lateral ventricles of man caused salivation, secretion of sweat, vasodilatation, vomiting and other effects which CUSHING (1932)

thought to be due to the stimulation of a parasympathetic centre in the hypothalamus. Acetylcholine, injected into the lateral ventricle of the cat causes a sleeplike state (DIKSHIT 1935). Pilocarpine applied in the lateral ventricle of the rabbit causes dilatation of the pupil, exophtalmus and hyperthermia (LIGHT et al. 1933). In man, acetylcholine or eserine injected into the lateral ventricles causes vomiting, increased peristalsis, sweating and, in some instances, a sleeplike state (HENDERSON and WILSON 1936).

In experiments on cats the present authors observed that the injection of acetylcholine into the hypothalamic region caused a cessation of breathing. This effect did not occur regularly; this may be due in part to the difficulty of making the injection into the same region in every experiment, and in part to the difficulty of finding the right dosage. In our experimental attempts to contribute to the problems under discussion we have applied the stable drugs eserine or prostigmine, and have performed the injections into the third ventricle in order to secure greater constancy of the regions reached by the drugs.

It is well established that visceral organs can be affected from the hypothalamic region. Sympathetic centres have been found there, and their electric stimulation may cause vasoconstriction, inhibition of the motility of the gut, sweating, piloerection, dilatation of the pupil, contraction of the nictitating membrane. The presence of a parasympathetic centre, originally suggested by CUSHING, was more recently claimed by BEATTIE et al. (1932, 1934), and HESLOP (1938), who observed that electric stimulation of definite hypothalamic nuclei causes fall in blood pressure, secretion of gastric juice, increased tone and motility in the digestive tract and the bladder, myosis etc. Some observers, however, were unable to obtain evidence of a parasympathetic hypothalamic centre (see RANSON and MAGOUN 1939).

Experiments.

Our experiments were performed on cats.

Anaesthesia. In every instance chloralose was used as an anaesthetic. The choice of anaesthetic is rather important in experiments of this kind. Barbiturates are likely to be adverse for the following reason: they exert a depressing action on the hypothalamus (MASSERMAN 1937); because of their atropine-like effect (see EMMELIN 1941) barbiturates should be omitted when studying proceedings where cholinergic nerves are involved. In a study on parasympathetic effects elicited from the

hypothalamus, BEATTIE and SHEEHAN (1934) notice that such effects are more regularly observed with chloralose than with dial. FULTON (1932) stresses that barbiturates should be omitted in experiments on the hypothalamus, and RANSON (1939) comments that sympathetic effects can well be elicited from the hypothalamus under barbiturates. It is interesting to note in this connection that in RANSON's experiments sweating could not be induced from the hypothalamus under nembutal. It was recently demonstrated that the sweat glands, the nerves of which are known to be cholinergic, do not respond to nervous stimulation when the cat is anaesthetized by a barbiturate (EMMELIN 1941).

Technique of dissection and injection. The method should, if possible, satisfy the following claims: 1) the brain should be left intact as far as possible; 2) the full amount of the injected fluid should with certainty reach the third ventricle; 3) the injected substances should not reach other centres than those related to the third ventricle.

Because of its topography and narrow frontal diametre the third ventricle is difficult to approach by direct injection. In attempts to bring active substances in contact with the hypothalamus, previous investigators injected via the lateral ventricles, the fourth ventricle (see RANSON and MAGOUN 1939) or via the aqueduct of Sylvius (LAFORA 1931). In our experiments the base of the skull was opened and the region between optic chiasm and the hypophysis exposed. The injection was performed with a capillary glass tube introduced into the third ventricle through the stalk of the hypophysis, as described later in detail.

The dissection was performed as follows: The anaesthetized cat was placed on its back, with the head slightly flexed towards the chest. A cannula was inserted into the trachea, both lingual arteries ligated and the entire floor of the mouth removed. In the base of the skull a hole is drilled, the borer being directed towards a point a few millimetres rostral to the suture between the sphenoid and presphenoid bones, which is situated approximately at the connecting line between the two pterygoid processes. During the boring, first the sphenoidal sinus is opened, and then the region between the anterior pole of the hypophysis and the optic chiasm. It is essential that the boring takes place in the middle line and not further rostral than in the region of the optic chiasm. The dura is cut. Bleeding can be avoided if the dissection is adapted carefully to the topography and a thermocauter used when removing the floor of the mouth. With correct boring the infundibulum can be exposed without bleeding. The dissection can be done in 45 minutes.

In the cat the third ventricle extends as far as to the infundibular process of the hypophysis (nomenclature according to RIOCH, WISLOCKI et al. 1940). Within the region of the neural stalk its wall is extremely thin and its frontal diametre somewhat larger. This part does not belong to the hypothalamus. If a glass capillary is inserted into the stalk of the hypophysis just rostral to the anterior pole of the hypophysis the third ventricle will be reached without damage to the hypothalamus. By very slow injection it is in our experiments possible to perform the injection in such a way that it does not extend beyond

Fig. 1. Photograph of the optic chiasm, infundibulum and hypophysis after the skull has been opened. The arrow points to the stalk of the hypophysis at the point where the injection is to be made.

the third ventricle. Figure 1 shows the exposed infundibulum situated between the optic chiasm and the hypophysis. The pars distalis of the hypophysis, which is lighter than the environment, is conical in the rostral part and points towards the neural stalk, which is grey. The arrow in figures 1 and 3 indicates the point where the injection was made. In the first series of our experiments, where the injection was made into the hypothalamus, the glass capillary was introduced through the optic chiasm.

The injection was performed by a glass capillary, 0.2—0.3 mm in diametre, attached to a pipette, where two cm height corresponded to 0.005 ml. The rate of injection is controlled by a screw and a tap. The capillary is mounted on a stand, fitted with a device for delicate adjustments in all three dimensions. By means of this device the capillary is introduced through the neural stalk into the third ventricle. We use a lens magnifying 10 times and an appropriate illumination. On injection the head of the cat must be perfectly fixed and flexed towards the chest in such a way that the connecting line between the anterior pole of the hypophysis and the optic chiasm is approximately horizontal. As a rule 0.005 ml was injected during $^1/_2$—1 minute.

At the end of the experiment the hypothalamic region with the hypophysis attached were removed, fixed in Carnoy's solution and embedded in paraffin. The position of the capillary was controlled by examining serial sections stained with haematoxylin-eosin and cut at 12 μ under the microscope. It is difficult to detect the insertion channel if the capillary is removed before fixation. We have achieved better results by applying the Carnoy solution to the infundibulum while the animal is alive, and by killing the animal five minutes later. By this method it is possible to

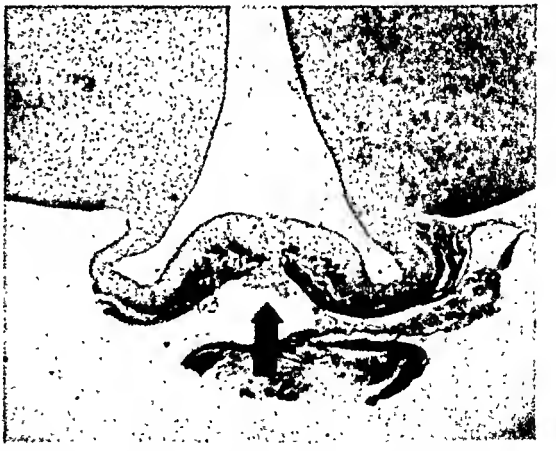


Fig. 2. Photomierograph of a frontal section through the hypothalamus of the cat at the level of the median eminence. The arrow points to the channel where the cannula had been inserted. Section cut at 12 μ. Hematoxylin-eosin stain. Magnification 20.

keep the channel open after the removal of the capillary. Fig. 2 represents a section through the insertion channel. In this figure there is no bleeding or tissue damage. In 16 experiments of this kind the microscopic pictures agreed with fig. 2. In two experiments there were traces of blood in the third ventricle. In two other animals it was found in the microscopic control that the capillary had not been inserted into the third ventricle. In these two experiments the typical peripheral effects obtained with injections into the third ventricle did not occur. With sufficient experience, the operator is well aware on inserting the capillary whether it takes the right or wrong course.

Fig. 3 indicates which regions are reached by the injection. In this and two other instances, 0.005 ml of a 0.5 per cent aqueous solution of gentianviolet were injected as a control. The animal was killed 10—15 minutes after the injection, that is, the same time within which the peripheral effects studied in this paper occur, and the brain examined. The lateral and the fourth ventricles remain white, while the entire walls of the third ventricle show a dark violet colour. The aqueduct of Sylvius shows a colouring which decreases and is absent at the entrance to the fourth ventricle. The infundibulum and the hypophysis remain uncoloured, which indicate that there is no retrograde leakage in the injection channel. The advantages of gentianviolet in experiments of this kind is discussed by BIETER et al. (1936).

Fig. 3. Photograph of a median section of the cat's brain after staining the third ventricle. The third ventricle and the aqueduct of Sylvius are stained. The arrow shows the point of injection.

Registered effects. The breathing was registered by means of a Marey's tambour attached to a tracheal cannula. The intervals between the respiratory movements were more accurately registered by the Fleisch "Ordinatenschreiber", where the heights of the ordinates are proportional to the lengths of the intervals. The impulses operating this instrument are derived from a special Marey tambour attached to the tracheal cannula.

The blood pressure in the carotid artery was registered by a U-shaped mercury manometer.

Intestinal motility was registered by a rubber balloon, inserted into the gut and attached to a U-shaped manometer, filled with water.

Gastric juice was obtained from a cannula inserted through the wall of the stomach. The juice was continually collected, its volume and acidity determined every 15 minutes.

The motility of the bladder was examined by inserting a cannula, which was attached to a membrane manometer. The cannula was

inserted through the urethra, the urine discharged, and suitable amounts of warm Tyrode solution introduced into the bladder.

Results.

Experiments with acetylcholine.

In the first series of experiments acetylcholine was injected into the anterior region of the hypothalamus. Fig. 4 demonstrates the inhibition of duodenal motility and tone, caused by the injection of 50 γ acetylcholine. Within five minutes after the injection the respiration ceases entirely, causing the death of the animal.

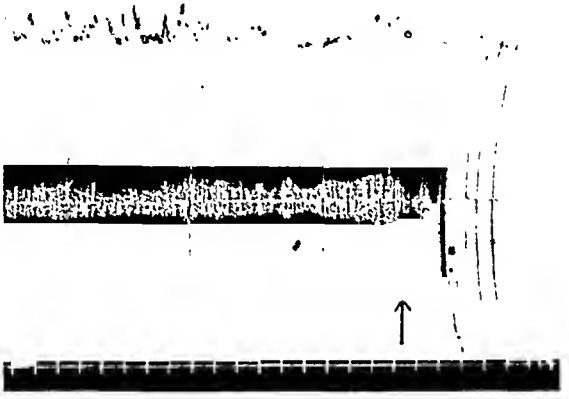


Fig. 4. Action of acetylcholine on respiration and intestinal motility after injection into the anterior region of the hypothalamus. From above: duodenal motility; respiration; time in minutes. At the arrow 50 γ acetylcholine were injected.

With smaller doses (10—20 γ) the apnoea was transitory. On injecting 50 γ acetylcholine intravenously into the cat no effects on intestinal motility and respiration were observed. On increasing the intravenous injecting dose to 1—2 mg acetylcholine apnoea of short duration was observed; the motor effect on the gut was stimulating. Considering these facts and the destroying activity of choline esterase, it is highly unlikely that the effects described should have been elicited in other ways than by acetylcholine acting on some cerebral centres.

Experiments with prostigmine and eserine.

We used prostigmine methylsulphate and eserine salicylate, dissolved in a 0.9 per cent NaCl solution. 50 γ were injected, dissolved in 0.005 ml. The insertion of the cannula into the third ventricle does not influence the respiration, or the intestinal or

bladder motility; a slight, transitory fall in blood pressure occurred. Injections of saline solution as a control caused no effect on respiration, bladder and intestine.

In a series the effect of prostigmine and of eserine on intravenous injection was controlled. Fig. 5 represents an experiment with prostigmine: prostigmine increases intestinal tone and the rate of gastric secretion and decreases the amplitude of the respiratory movements, whereas the rate of these movements is unaltered (or slightly increased in some experiments). The bladder motility,

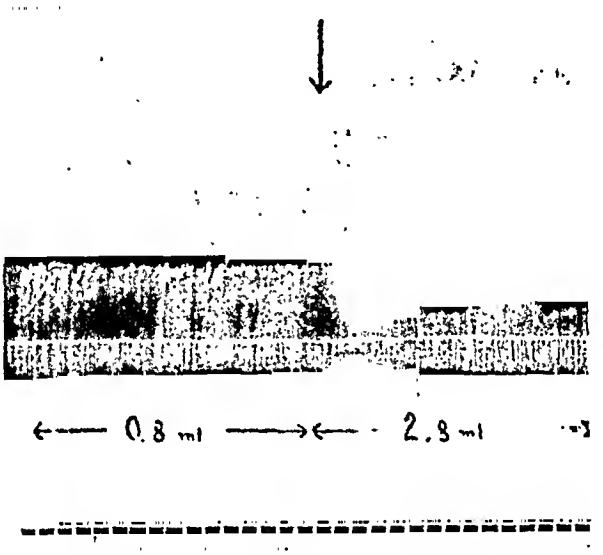


Fig. 5. Intravenous injection of prostigmine. From above: jejunal motility; respiration; volumes of gastric juice secreted in 15 minutes; time in minutes. At the arrow injection of 50 γ prostigmine methylsulphate into the femoral vein.

which was not registered in the experiment of fig. 5, was regularly stimulated by prostigmine.

Effect on respiration. On intraventricular injection both prostigmine and eserine caused a typical effect, which is demonstrated in figures 6 and 7. Within a few minutes after the injection the respiratory movements increase in size and decrease in rate. 15—30 minutes after the injection a complete apnoea develops. During this period the ventricles of the heart beat vigorously and without any palpable disturbances in rhythm. If artificial respiration is not instituted, or other action taken, the animal will not recover from the apnoea. If artificial respiration is maintained during some two hours, the animal regains the capacity of spontaneous breathing. The breathing can also be restored by repeated intravenous

injections during 1—2 hours of analeptic drugs, such as metrazol or nicethamide (fig. 8). This figure also demonstrates the effect of intraventricular injection of prostigmine on the blood pressure. If afferent impulses from the lungs are blocked by vagotomy central injection of prostigmine or eserine still inhibits respiration.

Effect on intestinal motility. Prostigmine and eserine injected centrally inhibit motility and tone of the small intestine (see fig. 6). This effect sets in about 8—10 minutes after the injection and persists during 15—20 minutes.

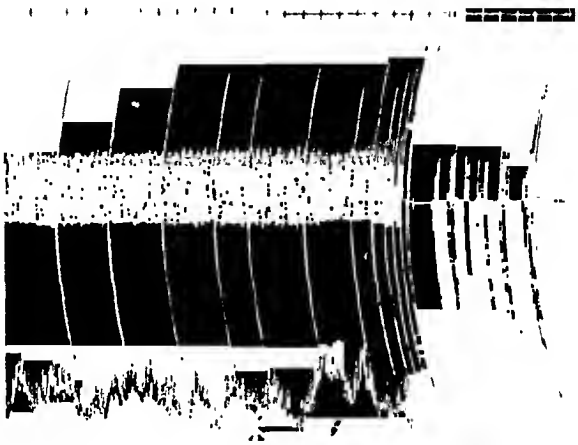


Fig. 6. Eserine injected intraventricularly. From above: time in minutes; respiration; jejunal motility. At the arrow injection of 50 γ eserine salicylate into the third ventricle.

The fact that the effect of prostigmine and eserine when injected into the third ventricle is opposite to that observed on intravenous injection indicates that the inhibition of the gut is induced by impulses from the brain. The effect is not brought about by the vagi, since it persists after section of both vagi in the neck. In a series of experiments the coeliac ganglion and the suprarenals were removed. In these animals intraventricular injections of prostigmine or eserine did not cause inhibition of the gut although the typical effect on respiration and bladder could be observed (fig. 7). These experiments suggest that eserine and prostigmine stimulate a sympathetic centre in the midbrain.

Effect on the bladder. Prostigmine and eserine injected intraventricularly strongly inhibit the motility of the bladder (see fig. 7).

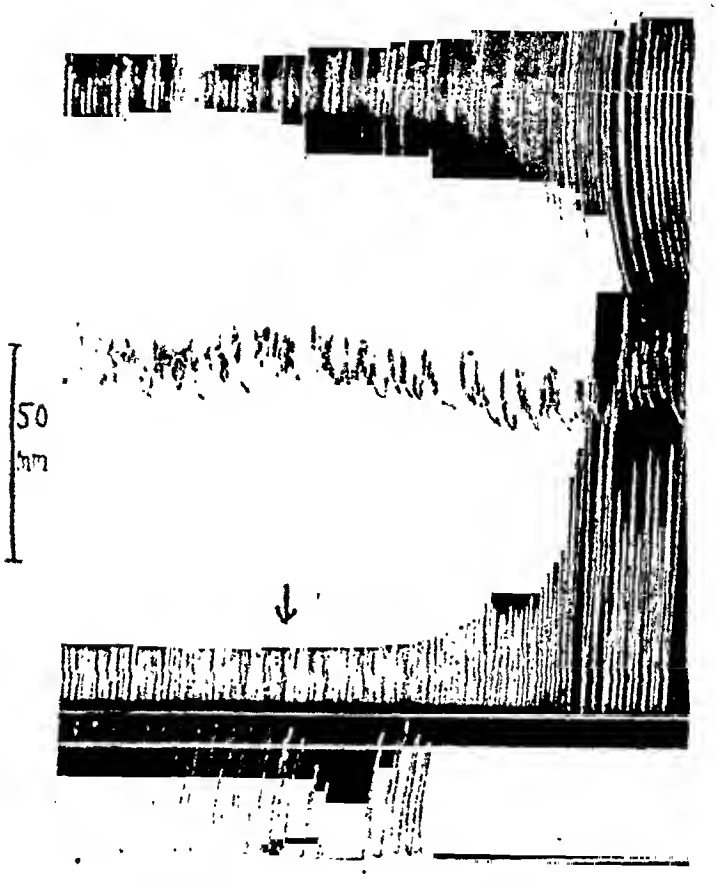


Fig. 7. Injection of prostigmine into the third ventricle after the coeliac ganglion and the suprarenals have been removed. From above: time in minutes; respiration, registered with a Marey's tambour; jejunal motility; rate of respiration, registered with the "Ordinatenschreiber"; the zero-line of the "Ordinatenschreiber"; signal; bladder motility. One mm of the ordinate in the original corresponds to 0.5 sec. At the arrow injection of 50 γ prostigmine methylsulphate into the third ventricle.

This action must obviously be of central origin since these substances, when injected intravenously stimulate the activity of the bladder.

Effect on the circulation. Intraventricular injections of prostigmine and eserine cause a slow and prolonged fall in blood pressure as measured in the carotid artery (see fig. 8).

Effect on gastric secretion. Eserine and prostigmine when injected intraventricularly do not augment the rate of gastric secre-

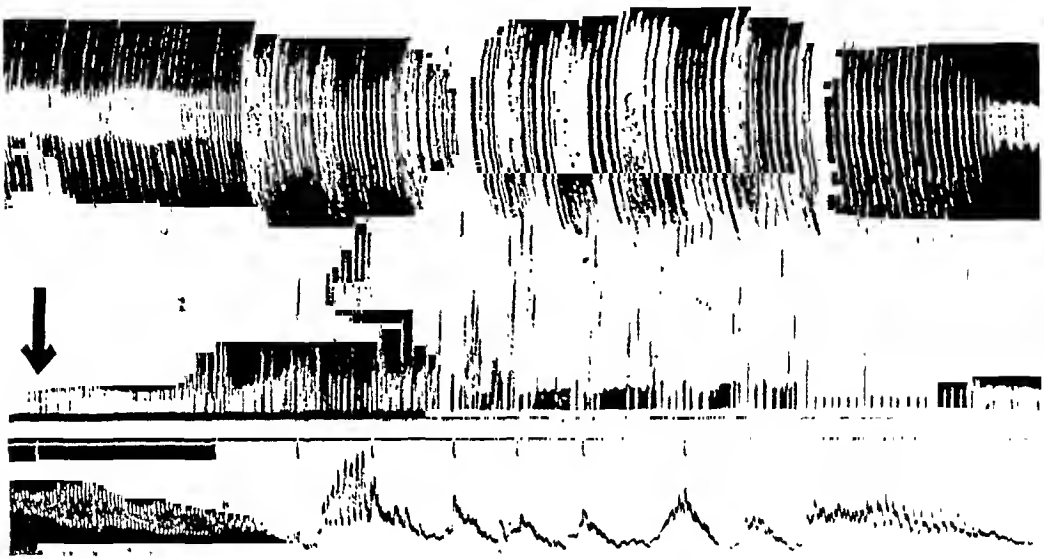


Fig. 8. Action of nicethamide during the apnoea caused by intraventricularly injected prostigmine. From above: time in minutes; respiration, registered with a Marey's tambour; rate of respiration; zero-line of the "Ordinatenschreiber", signal; blood pressure in the carotid artery. At the arrow injection of 50 γ prostigmine methylsulphate into the third ventricle. As soon as the typical respiration disturbances occur injection of 50 mg nicethamide intravenously. The injection was repeated when respiration disturbances began, in all seven times (at the marks following the arrow).

tion. On the contrary, there is a tendency for the volume of secreted juice to decrease. In some experiments it was observed that spontaneously secreted juice, which before the injection gave a positive reaction, was Congo negative after the injection.

Effect on body temperature. It is stated that intraventricular injection of pituitrine and other drugs are capable of influencing body temperature (LAWRENCE and DIAL 1932, LIGHT et al. 1933). In our experiments there was no significant change in the rectal temperature after injections of eserine and prostigmine into the third ventricle.

Effect on salivary secretion. Although the rate of secretion was not accurately measured it was obvious by inspection that the secretion of saliva was stimulated after intraventricular injections of eserine or prostigmine.

Observations with atropine. We intended to examine whether the effects induced by intraventricular injection of eserine and prostigmine were influenced by subsequent intraventricular injec-

tions of atropine. In control experiments with atropine alone 5—10 γ/kg of this substance was injected into the ventricle. A strong inhibition of bladder and intestinal motility was observed within a few minutes, while the respiration was not affected. Since it was observed in other controls that 5—10 γ atropine/kg injected intravenously strongly inhibited bladder and intestine motility, it

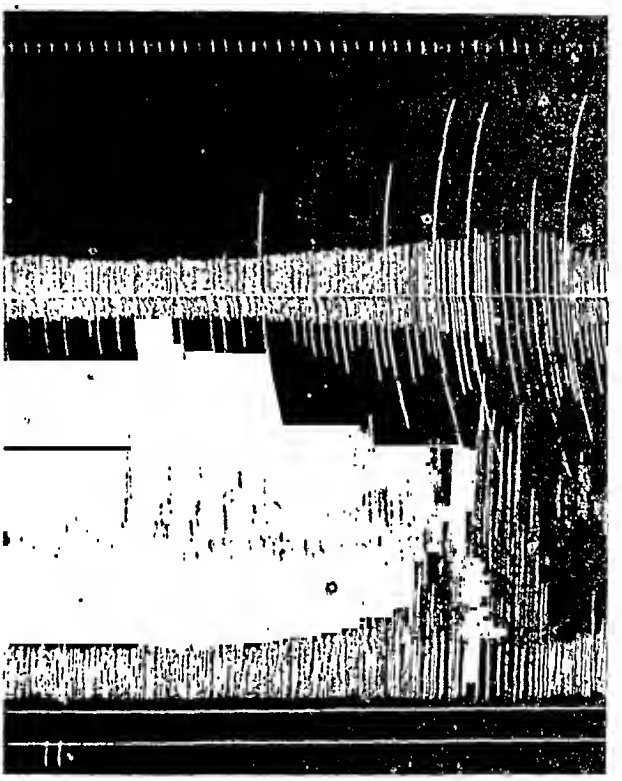


Fig. 9. Prostigmine suboccipitally. From above: time in minutes; respiration, registered with a Marey's tambour; jejunal motility; rate of breathing, registered with the "Ordinatenschreiber"; zero-line; signal. Between the marks on the signal-line 50 γ prostigminemethylsulphate were injected suboccipitally.

seems unsafe to state that this small dose of atropine inhibits the bladder and gut by a specific action on the midbrain, although it seems rather unlikely that the larger part of the atropine should within a few minutes be removed from the third ventricle to the blood. In our experiments no effects were observed with intravenous or intraventricular injection of less than 5—10 γ/kg. In view of this uncertainty and of the fact that atropine when injected intraventricularly acts like eserine and prostigmine on the gut and bladder, we have only investigated the effect of intravenously

injected large doses of atropine on the apnoea referred to previously. There was no typical effect of atropine on this apnoea apart from a tendency to prolong the period of breathing at a very slow rate if atropine was injected in time before apnoea occurred. SCHWEITZER and WRIGHT (1937) claim that eserine effects of central origin are not antagonized by atropine, whereas HENDERSON and WILSON (1936), MCKAIL et al. (1941), BÜLBRING and BURN (1941) have observed an antagonism of this kind.

Actions of suboccipitally injected prostigmine. In these experiments 50 γ prostigmine methylsulphate dissolved in 0.1 ml saline was injected suboccipitally. In two of four experiments this was followed by a decrease in the rate of breathing. The inhibition occurred later, and was slighter and more transitory than with injections into the third ventricle. The motility of the bladder was not influenced, whereas a slight stimulation was observed on the gut (see fig. 9). The effect on the gut occurred within a few minutes; this, together with the fact that the bladder was not influenced, suggest that the drug reaches the medullary vagal centres and stimulates them.

Discussion.

The actions observed after injection of prostigmine and eserine into the third ventricle are considered to be central in origin; they are opposite to those caused by intravenous injection. There are many indications that by the technique of injection employed in our experiments the active drugs are brought in contact with the hypothalamus. Controls with injection of coloured material show that during the time within which the studied effects occur the injected material does not extend beyond the third ventricle. The typical effects caused by injection of eserine and prostigmine into the third ventricle can not be elicited by suboccipital injection of the drugs. The assumption that, in our experiments, the hypothalamus is the main site of action is in agreement with the well-established fact that effects similar to those observed after intraventricular injections of eserine and prostigmine can be elicited by electrical stimulation of the hypothalamus. This causes an increased sympathetic discharge to the gut as described by BEATTIE (1932), BEATTIE and SHEEHAN (1934), HESLOP (1938), RANSON et al. (1939). According to these authors a centre is situated in the lateral hypothalamic area, which inhibits the motility of the

digestive tract. The motility and tone of the bladder is inhibited by electrical stimulation of the posterior region of the hypothalamus (BEATTIE and KERR 1936). The breathing can also be inhibited by electrical stimulation of the hypothalamus and adjacent regions (RANSON et al. 1939, LEITER and GRINKER 1934, ECTORS et al. 1934, HESS 1936). $CaCl_2$ injected into the infundibulum decreases the rate of breathing and may even cause apnoea (DEMOLE 1927, CLOETTA et al. 1930, 1934). In man respiratory disturbances in the rhythm of breathing have been observed in cases with tumours in the hypothalamic region (PENFIELD 1929, DOTT 1938).

In our experiments the depressing effect on breathing is counteracted by metrazol and nicethamide. This antagonistic action may in part be due to a stimulation of medullary centres; but it may be mentioned in this connection that these drugs stimulate breathing even when injected into the hypothalamus (MASSERMAN 1940).

A fall in blood pressure can be induced by electrical stimulation of definite hypothalamic regions (SILVER and MORTON 1936, HARE and GEOHEGAN 1939, BRONK 1933).

The parietal gastric glands can be excited by electrical stimulation of the lateral margin of the infundibulum (BEATTIE 1932). HESLOP (1938) demonstrated that electrical stimulation of supraoptic and tuberal regions was followed by the secretion of acid juice, while on stimulation of the mammillary region there was a tendency to decrease rate and acidity of secretion. In our experiments prostigmine and eserine, when injected into the midbrain, did not stimulate the secretion; on the contrary, there was a tendency to decrease the secretion rate and acidity.

Our experiments are not opposed to the view that acetylcholine plays a part in central excitation. In view of conflicting observations, it is of interest that eserine and prostigmine in our experiments exert identical effects. It is suggestive to hypothetize an identical mode of action for the two drugs: the choline esterase may be the point of attack. In our experiments acetylcholine when injected intraventricularly elicits similar effects as eserine and prostigmine. HENDERSON and WILSON (1936) observed that eserine and acetylcholine injected into the lateral ventricle in man elicit identical effects; they assume that eserine acts by inhibiting the esterase, thus favouring the accumulation of acetylcholine in active concentrations; they further assume that the acetylcholine is liberated by nervous impulses reaching the brain. In this connection it is of interest that, in our experiments, the actions of

acetylcholine are elicited immediately, and those of eserine and prostigmine not until 5—10 minutes after the injection.

There are further indications that acetylcholine is connected in some way or other with hypothalamic activity; MACINTOSH (1941) extracted acetylcholine from the hypothalamus, and in electrical stimulation of the hypothalamus ADAM et al. (1938) demonstrated acetylcholine in the outflow when the ventricular system was perfused.

Summary.

1. A method for injection of drugs into the hypothalamus and the third ventricle is devised.

2. Acetylcholine injected into hypothalamic regions has similar actions on the respiration and intestinal motility as those observed by other workers from electrical stimulation of definite hypothalamic regions.

3. Eserine and prostigmine injected intraventricularly cause apnoea, inhibition of the motility and tone of the gut and bladder, and some other effects which are similar to those described by other observers in electrical stimulation of hypothalamic regions.

4. It is suggested that acetylcholine, eserine and prostigmine excite hypothalamic cells, which constitute a sympathetic centre at this level.

References.

ADAM, H. M., R. A. MCKAIL, S. OBRADOR and W. C. WILSON, J. Physiol. 1938. *93*. 5 P.

BEATTIE, J., Canad. med. Ass. J. 1932. *26*. 400.

BEATTIE, J. and D. SHEEHAN, J. Physiol. 1934. *81*. 218.

BEATTIE, J. and A. S. KERR, Brain 1936. *59*. 302.

BIETER, R. N., R. W. CUNNINGHAM, O. LENZ and I. MCNEARNEY, J. Pharmacol. 1936. *57*. 221.

BÜLBRING, E. and J. H. BURN, J. Physiol. 1941. *100*. 337.

CHUTE, A. L., W. FELDBERG and D. H. SMYTH, Quart. J. exp. Physiol. 1940. *30*. 65.

CLOETTA, M. and H. FISCHER, Arch. exp. Path. Pharmak. 1930. *158*. 254.

CLOETTA, M., H. FISCHER and M. R. VAN DER LOEFF, Ibid. 1943. *174*. 589.

CUSHING, H., Papers relating to pituitary body, hypothalamus and parasympathetic nervous system, Springfield 1932.

DEMOLE, V., J. Pharmacol. 1927. *120*. 229.

DIKSHIT, B. B., J. Physiol. 1935, *83*. 42 P.

DOTT, N. M., Surgical aspects of the hypothalamus. In W. E. LE GROS CLARK et al.: The Hypothalamus, Edinburgh 1938.
ECTORS, L., N. L. BROOKENS and R. GERARD, Arch. Neurol. Psychiatr. Chicago 1938. *39.* 879.
EMMELIN, N., Acta Physiol. Scand. 1941. *2.* 289.
FLEISCH, A., Z. ges. exp. Med. 1930. *72.* 384.
FULTON, J. F., New Engl. J. Med. 1932. *207.* 60.
HARE, K. and W. A. GEOHEGAN, Amer. J. Physiol. 1939. *126.* 514 P.
HENDERSON, W. R. and W. C. WILSON, Quart. J. exp. Physiol. 1936. *26.* 83.
HESLOP, T. S., Ibid. 1938. *28.* 335.
HESLOP, T. S., Brit. J. Surg. 1938. *25.* 884.
HESS, W. R., Arch. Psychiatr. Nervenkr. 1936. *104.* 548.
KARPLUS, J. P. and A. KREIDL, Pflüg. Arch. ges. Physiol. 1910. *135.* 401.
LAFORA, G. R., An. Acad. méd.-quir. esp. 1931. *18.* 559, quoted from Ber. ges. Physiol. 1933. *70.* 555.
LAWRENCE, J. H. and D. E. DIAL, Proc. Soc. exp. Biol. N.Y. 1932. *30.* 49.
LEITER, L. and R. R. GRINKER, Arch. Neurol. Psychiatr. Chicago. 1934. *31.* 54.
LIGHT, R. U., C. C. BISHOP and L. G. KENDALL, J. Pharmacol. 1933. *47.* 37.
MACINTOSH, F. C., J. Physiol. 1941. *99.* 436.
MANN, P. J. G., M. TENNENBAUM and J. H. QUASTEL, Biochem. J. 1938. *32.* 243.
MASSERMAN, J. H., Arch. Neurol. Psychiatr. Chicago 1937. *37.* 617.
MASSERMAN, J. H., Effects of analeptic drugs on the hypothalamus of the cat; in D. RIOCH et al.: The Hypothalamus, Baltimore 1940.
MCKAIL, R. A., S. OBRADOR and W. C. WILSON, J. Physiol. 1941. *99.* 312.
NACHMANSOHN, D., Bull. soc. Chim. biol. Paris 1939. *21.* 761.
PENFIELD, W. G., Arch. Neurol. Psychiatr. Chicago 1929. *22.* 358.
RANSON, S. W. and H. W. MAGOUN, Ergebn. Physiol. 1939. *41.* 56.
RIOCH, D., G. B. WISLOCKI, J. L. O'LEARY, J. A. HINSEY and D. SHEEHAN, The Hypothalamus, Baltimore 1940.
SCHWEITZER, A. and S. WRIGHT, J. Physiol. 1937. *89.* 165.
SILVER, G. A. and H. G. MORTON, J. Pharmacol. 1936. *56.* 446.
WINKLER, F., Pflüg. Arch. ges. Physiol. 1908. *125.* 584.

From the Physiological Department, Karolinska Institutet, Stockholm.

A Search for Vagal Afferent Fibres Responding to an Increase of Pressure in the Central Veins.

By

BO GERNANDT and YNGVE ZOTTERMAN.

Received 5 October 1944.

In 1915 BAINBRIDGE proved that an increase in venous pressure caused a decrease in vagus tonus and an increase in accelerator tonus. Ever since BAINBRIDGE'S fundamental investigations in this field the manner in which the heart rate reacts to changes in the venous inflow, has been the subject of many investigations.

BAINBRIDGE carried out his experiments on dogs. He injected warmed blood or NaCl-solution at rectal temperature into the left ext. jug. vein; 200—400 ml. being injected in $1^1/_2$—4 min. or 40—50 ml in 15—30 sec. This produced a definite increase in the heart frequency. When a smaller amount was injected, the effect persisted for a shorter time.

The reflex was found to be absent when the acceleratory nerves of the heart were severed, and the suprarenal veins were ligatured. The effect was also dependent to some extent on the number of injections; at the beginning of the experiment the effect was greater and more lasting. The arterial pressure was practically constant during the experiments.

The result of BAINBRIDGE'S investigations have been very much discussed. Some authors were able to reproduce the results, others not. SASSA and MIYAZAKI (1920) repeated the experiments on frogs, rabbits, cats and dogs. They could not obtain any effect on frogs and rabbits, but on the other hand their experiments

on cats and dogs were successful. They used injections of NaCl-solutions, in order to increase the venous pressure; but in other experiments they achieved this by introducing a balloon into the right auricle. The reflex could also be produced when the balloon was inserted into v. cava sup. near the heart. The effect persisted, however, even after the sympathetic fibres had been severed.

In experiments on dogs DE GRAFF and SANDS (1925) were far from always able to produce any BAINBRIDGE reflex.

ANREP and SEGALL (1926) carried out experiments on innervated heart-lung preparations from dog and were able to elicit the BAINBRIDGE reflex. It was not obtained after severance of n. vagi, but remained unchanged after extirpation of the stellate ganglion. BAINBRIDGE considered that the increase in the venous pressure caused the reflex. ANREP and SEGALL pointed to other factors which are present with increased venous filling, namely, 1) increase in the diastolic volume of the heart, which affects the musculature of the heart and the visceral and parietal folds of the pericardium, 2) increase in the pulmonary pressure. These authors showed that the reflex remained unchanged after severance of the pulmonary ramifications of vagus and also after removal of the parietal fold of the pericardium.

In a histological investigation on rabbits, cats and dogs, NONIDEZ (1937) proved that areas could be shown within the intrapericardial part of the venae cavæ and the pulmonary veins containing numerous nerve terminals. He discusses the result of his investigations in the light of those of BAINBRIDGE, SASSA and MIYAZAKI, and arrives at the conclusion that the subendothelial nerve terminals are excited mechanically by the increased venous pressure. The finding of pressoreceptor structure in the central veins seemed to fit in very well with the existance of the reflex described by BAINBRIDGE.

The object of the present investigation was to study the activity within the afferent part of the BAINBRIDGE reflex by recording the action potientals set up in the vagus nerve by increased pressure within the large central veins.

Methods and results.

The experiments were performed on rabbits and cats weighing 2—3.5 kilos. The rabbits were anaesthesized with an intravenous injection of 7 cc. of a 20 % urethane solution per kilo, and the cats were

given 5 cc. of an 1 % chloralos solution intravenously. Cannulas were inserted in the usual way into the left carotid artery, the left femoral vein and the trachea. The venous pressure was observed by means of a piston recorder in the left jugular vein.

The electric response from the nerve was recorded by means of a resistance-capacity coupled amplifier and a cathode oscillograph previously described (ZOTTERMAN 1939).

In order to study the action potentials from afferent fibres running from the auricle or from the central veins, it is necessary to eliminate the action potentials in the afferent fibres of the vagus which run from the lungs and the respiratory tracts. For that reason we have made the following attempts. First we tried to separate the afferent fibres by splitting up the vagus nerve on the neck into a large number of fascicles in the hope to obtain fascicles containing the afferent fibres wanted but none from the lungs. This procedure failed entirely, as all the fascicles tested proved to contain afferent fibres from the lung. Another possibility would be to cut all fibres running to the bronchs as they leave the main vagus. The nerve fibres running to the lungs and to the heart, however, leave the main trunk of the vagus so close together that it seemed impossible to separate them. We also made several attempts to eliminate the afferent fibres to the lungs by crushing the walls of the right main bronchus by means of an arterial clamp but even this procedure did not succeed fully; there always remained a volley of impulses at each inflation of the lungs.

We then tried to eliminate the activity of these fibres by introducing 1—2 ml 10 % procain solution in the right bronchial system via an ureter cannula. This procedure greatly reduced the number of impulses set up at each inspiration but did not in any case eliminate them completely. As regards the rabbits, this method proved to be the only way of reducing the masking action of the lung afferents. In the cats, however, the thorax could be opened on the right side, which proved to abolish all the impulses from the right lung.

The venous pressure was raised by rapid infusion of 10—50 ml. body-warm Ringer solution into the left jugular vein, while recording from the vagus nerve. The effect was entirely negative however. In order to raise the signal-to-noise ratio of the potentials which we were searching for, the vagus nerve was divided into 5—10 fascicles, but in none of these we were able to find

the slightest sign of any increased activity following upon a sudden increase of the venous pressure. These negative findings were obtained in experiments upon altogether eight cats and twelve rabbits.

Discussion.

The failure to obtain any signs of afferent impulses following upon increase of the venous pressure raises the question whether the fibres are of such a small size that the method employed does not permit of their recording or whether there really exist in the right vagus afferent fibres reacting upon an increased venous pressure.

The recording of action potentials from fibres running from pressoreceptors of the aortic and carotid regions is a rather easy procedure. Thus we consider our negative findings rather significative. We have, however, to consider the possibility that the presumptive fibres from the heart are of such a small size that their activity cannot be traced, when the leads are placed on the whole nerve. It was for that reason that we split up the nerve in as many thin fascicles as possible in order to raise the signal-to-noise ratio. But even this procedure failed entirely, and it does not seem very likely that all the presumed pressoceptive fibres in the right vagus consist of only very thin fibres in the rabbit as well in the cat. And even if this were the case, we ought to have seen some signs of augmented activity when recording from very fine fascicles of the nerve when the venous pressure was raised suddenly.

The chemoreceptive fibres in the carotid sinus nerve of the dog were found to consist of very thin fibres (Euler and Zotterman 1942) but the pressoceptive fibres in the depressor nerve as well as in the carotid sinus nerve of the cat are of such a size that permits a fairly easy recording of their action potentials.

Thus we have failed in producing any direct evidence for the existence of these pressoceptive fibres in the right vagus nerve. A review of the literature shows that the indirect evidence hitherto supplied by experimental work is not unanimously positive. Thus, in experiments on dogs, De Graff and Sands could not always see any reflex, and when they found an effect, this persisted even after both vagi were cut. Thus the acceleration of the heart rate which follows upon an increase of the venous

pressure need not necessarily be due to a reflex elicited via afferent fibres from the central vein running whithin the right vagus trunc.

On the other hand, ANREP and SEGALL, who observed "that an increased output of the heart gives rise to reflex acceleration of the heart beat" in experiments with the innervated heart-lung preparation, many times could observe a definite acceleration with a minimum rise of the venous pressure, but in several cases the venous pressure did not rise at all or rose only for a short time. They thus considered the location of the receptor part of the reflex to the venae cavae to be doubtful and they also questioned whether the venous pressure is responsible for the reflex.

According to NONIDEZ, the extensive plexus between the openings of the venae cavae of the cat arises to one part from a nerve branch rising from the right vagus, and "large myelinated fibres" from this plexus are seen ending in the walls of the venae cavae. The nerve endings in the intrapericardial portions of the venae cavae were found to closely resemble the terminations in the carotid sinus and in the aorta, and were thus regarded as pressoreceptors. Such endings are not seen in the walls of the venae cavae of the rabbit, in which animal the BAINBRIDGE reflex cannot be demonstrated (SASSA and MIYAZAKI).

The negative result of our experiments demonstrates, we think, that the large myelinated fibres described by NONIDEZ cannot be afferent vagal fibres responding to a rise of the pressure in the central veins and the right auricle.

Summary.

1. Attempts have been made to record from the right vagus of cats and rabbits action potentials due to a sudden increase of the pressure within the central veins and the right auricle. The result was entirely negative.

2. This finding indicates that the nerve fibres serving as the afferent part of the BAINBRIDGE reflex are, if they really exist, of such a small diameter, that they cannot be recorded by the methods applied. This result is discussed in light of the previous experience of the BAINBRIDGE reflex presented in the literature.

References:

ANREP, G. V. and H. N. SEGALL, J. Physiol. 1926. *61.* 215.
BAINBRIDGE, F. A., J. Physiol. 1915. *50.* 65.
DE GRAFF, A. C. and J. SANDS, Amer. J. Physiol. 1925. *74.* 400.
EULER, U. S. v. and Y. ZOTTERMAN, Acta Physiol. Scand. 1942. *4.* 13.
NONIDEZ, J., Amer. J. Anatom. 1937. *61.* 203.
SASSA, K. and H. MIYAZAKI, J. Physiol. 1920. *54.* 203.
ZOTTERMAN, Y. Ibidem 1939. *95.* 1.

From the Chemical institution, Karolinska institutet, Stockholm.

Investigations on the Toxicity and Distribution in the Organism of the Copper (II) Ion in Respect of its Suitability as an Antidote against Hydrocyanic Acid.

By

CARL-JOHAN CLEMEDSON and BO NORBERG.

Received 14 October 1944.

The prime factor in hydrocyanic acid poisoning is generally supposed to consist in a blocking of enzyme systems important to the maintenance of life. Among the many methods that have been suggested for the treatment of cases of hydrocyanic poisoning (see WIRTH 1935), there seem to be only a few with a certain theoretical background. Treatment with reagents that bring about a conversion of the blood pigment to methemoglobin (ferrihemoglobin) aims at a neutralization of the hydrocyanic acid by forming the relatively stable and innocuous cyanmethemoglobin. Another principle is to neutralize the hydrocyanic acid by the addition of aldehydes or ketones, with which the hydrocyanic acid forms cyanhydrins (MARSHALL and ROSENFELD 1934). Experimental tests have, it is true, shown these methods to have a certain therapeutic usefulness, but from the theoretical point of view they are not above criticism. There seems, on the other hand, to be more theoretical support for the treatment proposed by AGNER (1939), consisting in the *intravenous* injection of copper (II) chloride. For enzymes whose effect is due to the presence of copper, the effect can be inhibited with hydrocyanic acid and restored by the addition of copper (KUBOWITZ 1938, MEIKLEJOHN and STEWART 1941, SKANSE and SUNDBLAD 1943). In the case of

mammals, the main point of attack for the hydrocyanic acid is, however, certainly the iron porphyry ferments. If, however, the cyanide is bound firmer to the copper than to the ferments, the copper will be able to inhibit the cyanide effect, as AGNER has demonstrated. The copper cyanide compound has been shown to be of a complex nature (NORBERG and JACOBSON 1945).

For the clinical application of AGNER'S copper treatment, a knowledge of the copper (II) ion's toxicity and its relations in the organism is of prime importance. As the data in the literature are in the first place lacking in detail and in the second place contradictory, they have been reinvestigated in the present paper. The copper-content in the blood and organs of rabbits has been determined after the injection of increasing amounts of copper (such as chloride or sulphate) in animals that have been killed a short time after the experiment and in animals that have been permitted to live for 20 hours or more after the injection. Liver and kidneys have been taken for the analyses, as the copper-content in these increases in case of acute copper poisoning (see e.g. EDEN 1940). In consideration of the fact that copper poisoning gives rise to cerebral symptoms (ESSER 1937 and others) and with an eye to the copper treatment of hydrocyanic poisoning, also the brain and the spinal cord were examined at first. For in the light of the rapid effect of the copper treatment of hydrocyanic poisoning it seems reasonable to assume that the copper (II) ion reacts with the hydrocyanic acid at the place where the latter takes effect. By means of copper analyses of various organs shortly after a copper treatment of hydrocyanic poisoning one should then be able to get an idea of whether the hydrocyanic acid effect is bound to any particular predilective site, or whether its distribution is more general.

In order to investigate these possibilities, rabbits were poisoned by the injection of hydrocyanic acid solution and treated with copper. In order if possible to accentuate any differences that there might be in the copper-content of the organs on the injection of copper alone and after copper-treated hydrocyanic poisoning, the hydrocyanic experiments were in several cases repeated after the animals had recovered from a previous poisoning with the help of the copper treatment.

And finally, some analyses were carried out on organs from healthy rabbits and on rabbits that had been killed with hydrocyanic acid without subsequent treatment.

Experimental Investigations.

Method.

The animals experimented upon have been exclusively young rabbits, weighing between 0.85 and 3 kg. The injections were made (except for 2 cases) without narcosis. For copper poisoning and copper treatment solutions of 0.03 mol copper chloride in 0.9 % NaCl and of 0.03 mol copper sulphate in 0.45 % NaCl respectively were used.[1] In connection with the injection of the copper solution, that was made at the rate of 1 ml during a period of about 5 seconds, no local reactions were to be observed — contrary to the experience of WALTHER and BEYER (1939). The probable explanation here is that these authors injected partly paravenously.

For the poisoning with hydrocyanic acid, an 0.0144 mol solution of hydrocyanic acid (potassium cyanide + an equivalent amount of HCl) in 0.9 % saline solution was slowly injected in one of the veins of the ear until unconsciousness and convulsions and the reduced respiratory and cardiac activity showed that the rabbit was moribund. At this point 1 ml copper solution per 4 ml hydrocyanic acid solution was injected in one of the veins of the ear. The rate of injection for the hydrocyanic acid solution was in general 1 ml for a period of 5—10 minutes, and in no case higher than 1 ml in 3 minutes. In this way it was possible to avoid an immediate collapse; and through the alternation of hydrocyanic acid and copper the rabbit could stand up to 3 or 4 times the minimum fatal dose of hydrocyanic acid. In the course of protracted experiments the hydrocyanic acid injections often gave rise to edema in the basal part of the ear, probably to a certain extent due to vessel contraction. In such cases the hydrocyanic acid injections were discontinued and the edema was massaged away. The letal dose of the hydrocyanic acid solution was 2—4 ml per kg of body weight (0.79—1.56 mg HCN per kg), which is in good agreement with the figures given by HUG (1932) for similar rates of injection.

In some cases the animals were poisoned with a current of hydrocyanic acid gas in a gas-chamber. The animals were killed a short time after the experiment, and copper analyses were carried out on heparinized blood and organs.

The *copper analyses* were carried out with the diethyldithiocarbamate method according to DELEPINE (BRAUN and SCHEFFER'S modification 1940). The organs were first dried to constant weight at 110°. Weighed amounts of the powdered organs were then incinerated with sulphuric acid and perchloric acid. Blood and plasma were combusted direct without previous drying. For the determination of the yellow copper complex, SUMMERSON'S photo-electric photometer with a 4.5 mm thick glass filter Schott BG 2 was employed. As the photometer value

[1] In the presence of 0.9 % NaCl a part of a copper is precipitated in $CuSO_4$, which is not the case with 0.45 % NaCl.

is not strictly proportional to the copper-content, the values of the samples were read off from a calibration curve. In table 1 are given some values for copper standard solutions according to this method. The mean error in the organ analyses was ± 10.4 % (σ), which was calculated from the double values according to FLEISCH (1926).

Table 1.

γ Cu/ml[1]	0	0.636	1.27	1.90	2.54	3.18	4.45	5.72
Mean photometer value	49.4	102.3	155.4	205.7	253.2	300.8	383	464
Standard deviation..	5.04	4.98	4.72	6.28	8.81	8.06	12.37	14.15

Experiments on Animals without Injection of Copper.

The data in the literature concerning the copper-content in different organs and in the blood vary greatly both for animals and for man, as may be seen from the compilation from various sources given on p. 124. This is probably due partly to the fact that different analytic methods have been employed, and partly to differences in diet. For this reason, copper analyses of blood and organs from normal, untreated rabbits were carried out. The results may be seen in table 2 A.

Table 2.

The copper-content in mg/kg fresh weight in some rabbit organs and in mg/litre blood. A: untreated animals. B: rabbits killed with HCN (gas-chamber).

No. of rabbit	Liver	Kidney	Brain	Spinal cord	Blood
A. 1	—	6.8	8.4	7.5	1.8
13	7.6	4.2	—	—	5.4
119	2.7	2.2	3.1	1.7	2.5
125	5.1	3.0	2.1	2.7	—
Mean	5.1	4.1	4.5	4.0	3.2
B. 306	1.13	3.30	2.74	1.80	6.4
307	3.36	7.15	2.3	1.94	—
309	3.0	3.06	1.30	—	—
311	4.10	3.40	2.90	—	1.88
314	3.90	3.0	2.30	—	1.95
Mean	3.1	3.98	2.31	1.87	3.41
Mean A+B	3.86	4.02	3.14	3.13	3.32

[1] The symbol γ means microgram (one millionth of a gram).

In order to ascertain whether hydrocyanic acid poisoning has any effect on the normal distribution of copper, copper analyses were carried out on some rabbits that had been poisoned to death with hydrocyanic acid in a gas-chamber. The results from this series of experiments are given in table 2 B. From these experiments we see that the normal distribution of copper in the organism is not noticeably changed in connection with hydrocyanic acid poisoning, since both series A and B show approximately the same variations in the copper values. The mean values for both series may thus be taken to represent the normal values for our animals.

The Toxicity of Copper (II) Compounds.

The minimum lethal dose with intravenous injection of copper (II) compounds (copper chloride, copper sulphate and glycocoll copper) in rabbits is by the majority of writers given as between 1 and 3 mg copper per kg of body weight (KOBERT, LEWIN, 1—2 mg, EDEN and GREEN 1.5 mg, WALTHER and BEYER 1.5—1.9 mg, BJERRUM and HENRIQUES, AGNER > 2 mg). The question has therefore been reinvestigated by us with doses of 1.5—6 mg copper per kg. The results are given in table 3.

Table 3.

Effect of different copper doses on rabbits.

Dose mg Cu/kg of body weight	1.5	2	2.5	3	4	6
Number of rabbits ...	1	9	13	5	6	4
» » dead	—	1	4	4	5	4
Time before death....	—	4 d.	6 h.–3 d.	3–14 h.	½–2 h.	10–35 min.
Number of surviving rabbits	1	8	9	1	1	0

Of the 4 animals that received 6 mg copper per kg, 3 died within 10 minutes. The fourth lived for 35 minutes. All showed the same symptoms: paralyses of the skeletal musculature; struggling respiration, the auxiliary respiratory musculature being called in; slow, weak and irregular cardiac activity, urination. Only in a few cases were clonic spasms observed before the paralysis set in. The copper analyses carried out on some of the animals that died or were killed within 1 hour are given in table 4.

Table 4.

Copper-content of blood and organs from rabbits killed within one hour after intravenous injection of copper salts.

No. of rabbit	Mg Cu/kg injected	Liver mg Cu/kg	Kidney mg Cu/kg	Blood mg Cu/l	Plasma mg Cu/l	Remarks
23.....	2.0	16.3	6.5	30.4	—	$CuCl_2$
24.....	»	13.9	12.0	27.9	14.4	»
28.....	»	15.3	10.5	20.2	26.2	$CuSO_4$. Killed after 5 min.
29.....	»	13.8	16.4	21.6	27.0	$CuSO_4$
Mean		14.8	11.4	25.0	22.8	
16.....	4.0	(8.9)	(7.8)	(8.4)	—	$CuCl_2$
25.....	»	25.5	33.0	48.9	33.7	»
26.....	»	23.0	22.7	46.7	—	»
30.....	»	20.5	16.2	31.0	38.2	$CuSO_4$
31.....	»	16.2	26.7	29.5	33.4	»
Mean		21.3	24.7	39.0	35.1	
18.....	6.0	14.1	17.4	29.6	—	$CuCl_2$. Lived 35 min
27.....	»	42.0	51.4	61.2	—	$CuCl_2$. Lived 5 min
32.....	»	27.5	28.7	52.9	53.9	$CuSO_4$. Lived 10 min
33.....	»	30.0	32.0	49.1	57.8	$CuSO_4$. Lived 8 min
Mean		28.4	32.4	48.2	55.9	

No. of animal	28	29	30	31	32	33
Hematocrit %	48	39	41	47	38	37

The copper values obtained in the case of animals killed after the lapse of more than 20 hours are to be found in table 5.

Table 5.

Copper-content of blood and organs of rabbits killed more than 20 hours after intravenous injection of copper chloride.

No. of rabbit	Mg Cu/kg injected	Liver mg Cu/kg	Kidney mg Cu/kg	Blood mg Cu/l
2.....	1.5	26.5	5.1	2.7
3.....	2.0	55.0	5.95	3.2
4.....	»	65.7	8.75	5.5
5.....	2.5	46.0	4.6	7.1
6.....	»	45.5	4.35	1.6
17.....	4.0	41.6	5.2	2.35

The copper-content of some organs of man in mg/kg fresh weight.[1]

Author	Liver	Mean value	Kidney	Brain
KLEINMANN and KLINKE (1929)	2.9—12.2	6.9		
Investigators cited by them	1.1—20.2		11.6	3.6—6.8
TOMPSETT (1935) and investigators cited by him	2.96—12.9		2.12—3.42	2.16—4.84
TUNG-PI CHOU and ADOLPH (1935)	18		8.2	
HAHN and FAIRMAN (1936)	0.6—17.5	6.1		
ALEXANDER and MEYERSON (1937)				10.9—11.4
EGGLETON (1940)	13		4.7	6—9.6

The copper-content of some organs of different animals in mg/kg fresh weight.[1]

Author	Liver	Kidney	Brain	Animal
Investigators cited by KLEINMANN and KLINKE (1929)	5.3—19			Various
HAHN and FAIRMAN (1936)	15—23	4—11		Dog
YOSIKOWA (1939)	7.5—10			Rabbit
EDEN (1940)	1—5	1—5	1—5	»
SÁROSDI (1940)	12			Cattle
SCHWAIBOLD and LESMÜLLER (1941)	0.45—12	0.24—0.8	0.3—0.48	Various

The copper-content of blood, serum or plasma of man in mg/litre.

Author	Whole blood	Serum or plasma
KLEINMANN and KLINKE (1929)	1.4—1.71	
Earlier investigators cited by them	0.42—1.44	0.62—2.11
GUILLEMET (1931)		0.56—0.75
TOMPSETT (1934) and investig. cited by him	1.85—2.38	1.83—2.45
NORINDER (1939)		3.07±0.095
SCHMIDT (1939)		1.10—1.40
EGGLETON (1940)	1.6—1.72	
BRAUN and SCHEFFER (1940)	1.19—1.57	
KEHOE, CHOLAK and STORY (1940)	0.25	0.25
Investigators cited by ÆXTRUP (1943)	1.32—1.55	0.7—1.38
DAHL (1943)		0.42—0.88
NILSSON (1944)		0.84—1.47

[1] For the calculation of these values when the copper content is given in dry substance we have used the mean values for dry substance obtained from our rabbits: 54 livers: 24.4 ± 6.04 %, 57 kidneys: 20.2 ± 3.56 % and 26 brains: 19.0 ± 0.9 %.

The copper-content of blood, serum or plasma of different animals in mg/litre.

Author	Animal	Whole blood	Serum or plasma
ELVEHJEM and coll. (1929)	Various	0.54	0.14
GUILLEMET (1931, 1932)	»	0.5—0.88	0.61—1.4
MC FARLANE (1932)	Pig, ox	1.63—2.26	
LOCKE and coll. (1932)	Rabbit		0.5—0.72
TOMPSETT (1934)	Various	1.39—2.23	1.61—2.17
YOSIKAWA (1939)	Rabbitt	0.9—1.0	
EDEN (1941)	Ewes	0.5—2.4	

It emerges from the tables that the copper-content in the brain and the spinal cord was not changed in relation to the normal values even by the big copper doses. This does not, however, contradict the assumption that the toxic effect of the copper may be due to injury to the central nervous system. For as PENTSCHEW and KASSOWITZ (1932) have shown, with suboccipital injection of copper (II) salts only 0.01—0.02 mg is sufficient to take fatal effect. Even if this amount of copper remains quantitatively in the brain, it is not analytically observable with the method employed by us. On the other hand, the symptomatology shown by PENTSCHEW and KASSOWITZ's animals diverges from that described above in so far as their animals first had a period of increased irritability with convulsions before paralysis and death occurred. It thus seems more probable that the toxic effect of the copper after intravenous injection is due to a general poisoning of the cells through the enzyme-inhibiting effect of the copper as a heavy metal, and not to a specific central nervous effect. That the poisoning sets in also in muscles has, moreover, been shown in isolated organs by HARNACK (1874).

The copper-content in the blood, the liver and the kidneys, on the other hand, is strongly increased if the animal has been killed within an hour after the injection of the copper. In blood and the kidneys, however, the copper-content sinks, so that after the lapse of twenty-four hours it is practically normal. At the same time the content in the liver rises sharply, so that one finds in the liver between 75 and 100 % of the amount of copper injected, as in some few cases has been found to be the case in man (see e.g. JOEST).

As regards the distribution of the copper between blood corpuscles and plasma, opinion is very much divided. Thus ELVE-

HJEM and his co-workers (1929) state that the copper-content in the blood corpuscles is double what it is in plasma; and SARATA (1934) finds 3—4 times as much copper in the blood corpuscles as in plasma (man, horses, rabbits, cattle); while GUILLEMET (1932), on the other hand, finds 5—6 times as much in plasma as in the blood corpuscles. TOMPSETT (1934), BJERRUM and HENRIQUES (1935) and HOLMBERG (1941) find an equal distribution. As the analyses in tables 4 and 6 show, one does indeed find a large part of the injected copper in the plasma, but also the blood corpuscles have taken up considerable quantities, in some cases even a good deal more than the plasma. In those cases in which the copper has been injected as sulphate, however, the copper-content in the plasma is always higher than in the blood corpuscles.

As the distribution of copper between blood and tissues changes with the time elapsing after the injection of the copper, and as the

Table 6.

Copper-content of blood and organs after copper chloride injections at different times before death. Total dose 4 mg Cu/kg body weight.

Plan of injections.

Rabbit No.	44 45	48 49	46 47	16 50	51 52
Time before death	Mg of copper per kg of body weight injected				
120 minutes	1	4	—	—	—
60 »	1	—	2	4	—
30 »	1	—	1	—	4
15 »	1	—	1	—	—

Analyses.

Rabbit no.	Liver mg Cu/kg	Kidney mg Cu/kg	Blood mg Cu/l	Plasma mg Cu/l	Hematocrit
44	18.3	42.1	35.5	25.1	55
45	13.0	28.8	34.4	34.0	53
48	22.7	25.2	27.1	15.6	55
49	21.1	26.6	18.5	18.4	57
46	14.0	30.0	43.8	38.8	—
47	15.3	32.8	35.5	33.8	—
16	(8.9)	(7.8)	(8.4)	—	—
50	23.2	31.0	28.1	32.6	45
51	23.5	21.0	33.7	35.0	44
52	21.7	31.0	34.8	41.6	42
Mean	19.2	29.8	32.4	30.5	

addition of the copper in several hydrocyanic acid experiments took place successively, a number of experiments were carried out in which only copper was injected, but in a succession of doses at intervals corresponding to those used in the hydrocyanic acid experiments. The results of the copper analyses are given in table 6.

It appears from this that the copper-content in the liver shows a considerably lower value if the injected amount of copper is distributed over several doses (rabbit 44—47), as occurred in the hydrocyanic acid experiments, than if the whole amount of copper is injected at once (rabbits 48—52 and table 4). The copper-content in blood tends to show higher values in the former case. In the kidneys the copper-content is about the same in both series.

Copper Treatment of Hydrocyanic Acid Poisoning.

These experiments comprise two series. In the one the animals have been permitted to live for twenty-four hours or more after the experiment before being killed. In the other series the animals were killed within 15—30 minutes after the treatment. Also the rabbits that owing to a too rapid injection of hydrocyanic acid or a too long delayed injection of copper did not recover, have been included. In the experiments in which a hyper-lethal dose of poison was aimed at it proved to be of particular importance not to hasten the poisoning too much, as it then became difficult to control. Apart from these "abnormal" poisonings, the experiments confirm, as regards the effect of the copper as an antidote, AGNER'S result.

The copper analyses from the series of hydrocyanic acid poisonings are given in tables 7 and 8. To illustrate the course of the ex-

Table 7.

Copper-content of blood and organs of animals poisoned with hydrocyanic acid and treated with copper injections and killed one day or more after the treatment.

Rabbit No.	Mg Cu/kg injected	Liver mg Cu/kg	Kidney mg Cu/kg	Blood mg Cu/l	Killed after days
9.....	1.4	21.3	3.9	9.4	2
10.....	2.0	50.4	3.3	1.6	12
11.....	2.0	29.7	4.2	1.05	1
20.....	3.15	31.2	4.4	1.57	1
22.....	3.9	48.8	4.0	8.5	1
15.....	4.7	65.2	4.77	3.6	1

Table 8.

Copper in blood and fresh organs of animals poisoned with hydrocyanic acid, treated with copper and killed within two hours after the last injection.

Rabbit no.	Mg Cu/kg injected	Liver mg Cu/kg	Kidney mg Cu/kg	Blood mg Cu/l	Plasma mg Cu/l	
38[1]	1.8	9.2	9.9	13.3	2.58	$CuSO_4$
7[1]	2.0	10.2	8.1	—	—	$CuCl_2$
8	»	9.75	6.3	16.6	—	»
34	»	16.5	16.7	23.7	—	$CuSO_4$
35	»	12.9	8.7	10.0	—	»
308	»	19.5	8.8	13.8	—	$CuCl_2$, gased
Mean		13.0	9.8	15.6		
12[1]	4.0	21.0	23.0	32.0	—	$CuCl_2$
301	»	28.8	22.1	—	—	»
21	»	19.0	11.9	19.5	—	»
42[2]	»	37.0	27.5	28.3	1.86	»
43[2]	4.2	36.0	26.6	(71.1)	3.78	»
Mean		28.4	22.2	26.6	2.82	
41[1]	2.7	26.2	21.1	6.6	3.9	$CuSO_4$
19[1]	2.8	24.8	12.7	2.1	—	$CuCl_2$
39	2.9	19.7	15.0	23.1	5.8	»
14	5.15	17.3	15.4	29.6	—	»

Rabbit no.	34	35	38	39	42	43
Hematocrit	56	50	34	46	59	49

periments where a hyper-lethal dose of hydrocyanic acid was injected, the data from one such experiment are given in table 9.

Table 7 shows that the distribution of copper after twenty-four hours is about the same as after the addition of copper alone (cf. table 5). From table 8 it emerges that the acute distribution of copper in the organism is different in the case of a copper-treated hydrocyanic acid poisoning from that observed where only copper is injected. The most striking difference refers to the *distribution of the copper in the blood.* While the pure copper injection gives an in the main equal distribution of copper between blood corpuscles and plasma ($Cu_{blood} : Cu_{pl} = 0.77 — 1.74$, mean 1.1), one finds after hydrocyanic acid poisoning a considerably more marked increase of the copper-content in the blood corpuscles than in the plasma, and this regardless of whether the treatment took place

[1] Animal died when the copper solution had just been injected.
[2] The experiment started with light narcosis by means of Narkotal »Astra».

Table 9.

Rabbit 12. 8/4 1948. Weight 1.4 kg.

Time minutes	Injected HCN solution ml	Injected $CuCl_2$ ml	Effect
3.....	1.0		
6.....	1.0		
8.....	0.6		Convulsions
9.....	0.2		Impetuous convulsions with tremor and nystagmus. After half a minute only tremor
16.....	0.87		Insignificant convulsions of short duration
19.....	0.83		Rapid worsening. Respiration irregular. Heart beats weak, frequency less than 30 for half a minuto
21.....			Rabbit sitting upright with paws stretched
24.....			Sitting in normal position
30.....	1.0		Regular convulsions during about 10 seconds with weak and irregular respiration and temporary slowing down of heart frequency
32.....			Looks normal again
37.....	0.7		Violent convulsions
37½.....		1.4	Heart activity immediately improving
38.....			Sitting in normal position
Pause for 22 minutes			
68.....	3.0		Tremor, no corneal reflex, short cry
71.....			Respiration better. Corneal reflex +
79.....	1.0		Slight convulsions
82.....	0.8		Convulsions. Corneal reflex +
85.....			No corneal reflex
85½.....		1.4	Respiration and heart activity ebbing out
88.....			Weak heart flimmer
90.....			Dead
Totally	11.0	2.8	

with copper chloride or copper sulphate ($Cu_{blood} : Cu_{pl} = 1.7 — 15.2$, mean 6.5). Further, the copper-content in the blood is after copper-treated hydrocyanic acid poisoning distinctly lower than it is found to be after a corresponding injection of copper alone. As regards the copper-content in the liver, the case is reversed, so that the hydrocyanic acid poisoned animals 12, 21, 42 and 43, that are most closely comparable with rabbits 46 and 47 (table 6), and 301, that should be compared with 48 and 49, have between

30 and 150 % more copper than those animals that were treated only with copper (table 10).

The copper-content in the kidneys shows less difference, and rather a tendency to lower values after hydrocyanic acid poisoning.

Table 10.

Copper-content in the liver after treatment: A. with copper alone and B. with hydrocyanic acid and copper in comparable experiments.

A.		B.	
Rabbit no	mg Cu/kg	Rabbit no	mg Cu/kg
44	18.3	12	21.0
45	13.0	19	24.8
46	14.0	21	19.0
47	15.3	41	26.2
		42	37.0
		43	36.0
Mean	15.6		27.3

Table 11.

Copper-content in mg/kg fresh organ. A: rabbits injected with $CuCl_2$ and killed within 2 hours. B: ditto but killed after one day. C: Animals injected with HCN solution and treated with $CuCl_2$, killed within 2 hours. D: ditto but killed after one day or more.

A. No	Cu	B. No	Cu	C. No	Cu	D. No	Cu
			Brain.				
16	3.8	2	3.3	7	3.13	9	2.95
18	2.3	3	3.6	8	3.3	10	4.8
		4	3.2	12	4.6	11	6.1
		5	3.7	14	2.26	15	2.5
		6	3.55	301	2.8	20	2.0
		11	2.06	19	2.6	22	2.33
				21	2.9		
				308	2.54		
Mean	3.05		3.2		3.0		3.45
			Spinal cord.				
16	2.9	2	3.05	7	5.0	9	3.4
18	3.4	3	2.6	8	4.55	10	4.1
		4	4.8	12	4.0	11	2.96
		5	3.4	14	1.76	15	2.12
		6	3.67	301	3.31	20	1.88
		11	2.06	19	2.54	22	1.82
				21	3.3		
				308	2.03		
Mean	3.15		3.3		3.3		2.72

The copper-content in the brain and spinal cord showed no significant differences between normal animals (table 2) and copper-hydrocyanic acid-treated or only copper-treated animals (table 11).

Discussion.

As regards the minimum lethal dose, the experiments show that rabbits can stand 2 mg copper (II) ions per kg of body weight, so that the statements in the literature referring to a lower tolerance-limit, i.e. greater toxicity, are probably exaggerated. As this dose is more than sufficient to neutralize the effect of a lethal amount of hydrocyanic acid, the risks entailed in the use of copper as an antidote to hydrocyanic acid seem to be definitely counteracted by the advantages. A further consideration is that in hydrocyanic acid poisoning a large part of the injected copper is bound in the form of a cyan-complex that owing to its low complex-constant can only have a negligible copper-ion effect (see table 7). The experiments did not afford any evidence that the body's own neutralization of hydrocyanic acid takes place with the help of the body's natural supply of copper. As some other mechanism must be active, one ought to be able to reduce the dose of copper and leave the neutralization of a small part of the hydrocyanic acid to the body's own natural forces (cf. HUG). If one thus reduces the therapeutic dose of copper to 1 mg copper per kg of body weight, one attains a negligible toxicity on the part of the antidote whilst retaining a good therapeutic effect. Experiments in this direction, however, have not been carried out, as the poisoning technique employed by us is scarcely applicable to the conditions that are of interest for human pathology.

The point of departure for the determination of the copper distribution in cases of copper-treated hydrocyanic acid poisoning has been the idea mentioned in our introductory remarks, of the possibility of thus getting on the track of the site in the organism where the hydrocyanic acid takes effect. The experiments have in this respect led to the following result: *that an acute enrichment of copper takes place in the liver, and a relative enrichment in the blood corpuscles,* as compared with the conditions after injection of copper alone. At the same time the copper-content of the blood is lowered in relation to corresponding experiments with copper alone. As the greater part of the injected amount of copper is found in the analyzed organs, especially in the liver, and in the

blood, it is probable that the quantitatively most important point of attack for the hydrocyanic acid is to be found in the liver and the blood. Since hydrocyanic acid is bound to ferriporphyry enzymes, and there is a plentiful supply of catalase precisely in the liver and the blood corpuscles (SENTER 1903, v. EULER 1934, AGNER 1944), our experiments favour the assumption *that the most important effect of the hydrocyanic acid from the quantitative point of view consists in an inhibition of the catalase.* A small part of the hydrocyanic acid probably inhibits the catalase and other iron porphyry ferments (peroxidases etc.) in the other organs which qualitatively must be of great importance (cf. symptoms from the central nervous system).

Summary.

By means of copper analyses of blood and organs from rabbits that have been given intravenous injections of copper (II) salts in varying doses, and from rabbits that before this treatment have been poisoned to death with hydrocyanic acid, it has been shown 1. that the minimum lethal dose in the case of copper is over 2 mg per kg of body weight, 2. that copper is enriched in the liver and the blood corpuscles after copper-treatment of hydrocyanic acid poisoning and 3. that the copper cyan-complex has a lower toxicity than copper.

On the strength of these results it may be said that the use of copper (II) salts in a dose of 1 mg copper per kg body weight can safely be prescribed as an antidote against hydrocyanic acid according to AGNER's technique (intravenous injection). The toxicity of the copper is presumed to be due to an inhibition of enzyme-systems of vital importance to the organism. In the light of our experiments it is probable that the quantitatively most important mechanism of the hydrocyanic acid effect consists in an inhibition of catalase in blood corpuscles and liver.

Bibliography.

AGNER, K., Naturwissenschaften. 1939. *27.* 31.
AGNER, K., Ark. Kemi. Mineralogi, Geologi 1943. *17 B.* no. 9.
ALEXANDER, L., and. A. MEYERSON, Trans. Amer. Neurol. Ass. 1937. *63.* 109. Chem. Abstr. 1938. *31.* 5487.
AXTRUP, S., Sv. läkartidn. 1943, *40,* 2953.
BJERRUM, J., and V. HENRIQUES, Scand. Arch. Physiol. 1935. *72.* 271.
BRAUN, L., and L. SCHEFFER, Biochem. Z. 1940, *304.* 397.

DAHL, S., Nord. Med. 1943. *20*. 2389.
EDEN, A., and H. H. GREEN, J. Comp. Path. Ther. 1939. *52*. 301. Chem. Abstr. 1940. *34*. 2922.
EDEN, A., ibidem 1940. *53*. 90. Chem. Abstr. 1940. *34*. 5533.
EDEN, A., Biochem. J. 1941. *35*. 813.
EGGLETON, W. G. E., Chin. J. Physiol. *1940*. *15*. 33. Chem. Abstr. 1940. *34*. 4780.
EGGLETON, W. G. E., Biochem. J. 1940. *34*. 991.
ELVEHJEM, C. A., H. STEENBOCK, and E. B. HART, J. Biol. Chem. 1929. *83*. 21.
ESSER, A., Dtsch. Z. ges. gerichtl. Med. 1937. *27*. 253.
VON EULER, H., Chemie der Enzyme 1934. *2*. III. 1.
FLEISCH, A., Biochem. Z. 1926. *177*. 375.
GUILLEMET, R., C. R. Soc. Biol. Paris 1931. *108*. 32 and 1932. *109*. 652.
HAHN, P. F., and E. FAIRMAN, J. Biol. Chem. 1936. *113*. 161.
HARNACK, E., Arch. exp. Path. Pharmak. 1874. *3*. 46.
HOLMBERG, C. G., Acta physiol. scand. 1941. *2*. 71.
HUG, E., C. R. Soc. Biol. Paris. 1932. *111*. 87.
JOEST, W., Sammlung von Vergiftungsfällen. Berlin 1937. *8*. A. 99.
KEHOE, R. A., J. CHOLAK and R. V. STORY, J. Nutrit. 1940. *19*. 579.
KLEINMANN, H., and J. KLINKE, Virchows Arch. 1929. *275*. 422.
KOBERT, R., Lehrbuch der Intoxikationen, Stuttgart 1906.
KUBOWITZ, F., Biochem. Z. 1938. *296*. 443.
LEWIN, L., Gifte und Vergiftungen, Berlin 1929.
LOCKE, A., E. R. MAIN, and D. O. ROSBASH, J. Clin. Invest. 1932. *11*. 527.
MARSHALL, E. K., and M. ROSENFELD, J. Pharmacol. 1934. *52*. 445.
MCFARLANE, W. D., Biochem. J. 1932. *26*. 1022.
MEIKLEJOHN, G. T., and C. P. STEWART, Biochem. J. 1941. *35*. 755.
NILSSON, F., Nord. Med. 1944. *21*. 278.
NORBERG, B. and B. JACOBSON, to be published.
NORINDER, E., Uppsala läkarefören. förhandl. 1939. *45*. 5.
PENTSCHEW, G., and H. KASSOWITZ, Arch. exp. Path. Pharmak. 1932. *164*. 667.
SARATA, U., Jap. J. med. Sci. Biochem. 1934. *2*. 305. Chem. Abstr. 1934. *28*. 6801.
SÁROSDI, J., Chem. Abstr. 1940. *34*. 2432.
SCHMIDT, H. G., Biochem. Z. 1939. *302*. 256.
SCHWAIBOLD, J., and A. LESMÜLLER, Vorratspflege u. Lebensmittelforsch. 1941. *4*. 100.
SENTER, G., Z. phys. Chem. 1903. *44*. 257.
SKANSE, B., and L. SUNDBLAD, Acta physiol. scand. 1943. *6*. 37.
SUMMERSON, W. H., J. Biol. Chem. 1939. *130*. 149.
TOMPSETT, S. L., Biochem. J. 1934. *28*. 1544 and 1935. *29*. 480
TUNG-PI CHOU and W. H. ADOLPH, Ibidem. 1935. *29*. 476.
WALTHER, R., and K. BEYER, Biochem. Z. 1939. *301*. 315.
WIRTH, W., Arch. exp. Path. Pharmak. 1935. *179*. 558.
YOSIKAWA, H., Jap. J. Med. Sci. Biochem. 1939. *4*. 211. Chem. Abstr. 1940. *34*. 6688.

From the Biological Institute of the Carlsberg Foundation, Copenhagen.

Protein Metabolism of Tissue Cells in vitro.

2. Accessory Growth Substances in Animal and Vegetable Tissues.

By

TAGE ASTRUP, ALBERT FISCHER and MOGENS VOLKERT.

Received 23 October 1944.

In a previous work (FISCHER 1941 a) tissue cells *in vitro* were shown to be unable to utilize the protein contents of dialyzed culture media for their own metabolism. Both the maintenance and the growth of the cells require the presence of dialyzable substances of low molecular weight. These substances are present in untreated serum and plasma, while they are removed from dialyzed serum and plasma, and hence the tissue cells will perish on culture media treated in this way. It was found practicable to supplement to some extent the dialyzed media with fractions of acid-hydrolyzed or trypsin-digested protein. But studies on the effect of various amino acids and mixtures of such showed that the mixture of ten amino acids found by ROSE and collaborators (1938) to be necessary for the organism had no effect on the tissue cells grown on dialyzed media. On the other hand, a fairly good effect was obtained with the amino acids found by BERGMANN and NIEMANN (1936) to be present in fibrin. Among these amino acids, cystine was found to be the one on which the effectivity of the mixture depended. When cystine was removed from the mixture, the entire rest was quite ineffective, whereas cystine alone had a distinct supplementary quality, which was further increased by addition of the rest of the amino acids.

It was found impossible with mixtures of various amino acids to supplement the dialyzed media to such an extent that they became as effective as the genuine, non-dialyzed media. And after more thorough dialysis — for removal of the remnants of the dialyzable substances from the media employed —, the complementary capacity of the amino acid mixture was further reduced even though it was still noticeable in relation to the control culture. It was found, however, that a boiled extract of kidney was able almost completely to replace the substances removed by dialysis (FISCHER and ASTRUP 1942). The active substances present in this extract were able to stand autoclaving, and they were dialyzable. By fractionation, mixtures were isolated which in themselves were less active but still able to supplement the amino acid mixture of BERGMANN.

Studies on the complementary capacity of proteins broken down by means of pepsin and erepsin (FISCHER 1942 a) indicate that the higher cleavage products are more effective than the lower ones when homologous proteins are employed, whereas the reverse is the case on employment of heterologous substances.

Numerous experiments have been carried out since, in order to obtain suitable raw material for a more precise characterization of the accessory substances, and these studies will be reported in this paper.

A. Technique.

In its main features the technique here employed is the same as described previously (FISCHER 1941 a; 1942 a), FISCHER and ASTRUP (1942). The work was carried out consistently with double dialysis of the plasma and serum in order completely to avoid the effect of the complementary dialyzable substances present in the plasma and serum. The embryonic extract was dialysed only once. As a rule, periosteal fibroblasts were employed — from the frontal bone of 14-day old chick embryos. Deviations from the usual technique will be evident from the remarks concerning the individual experiments.

B. Experimental.

1. Kidney Extract.

The previous studies on boiled extract of kidney (FISCHER and ASTRUP 1942) showed that the active substances in the mother-liquor from the treatment with fullers earth do not lend them-

selves readily to further separation. The various fractions all turn out to be more or less active and are completed by the Bergmann mixture without any distinct difference between the various fractions. Treatment with cupric hydroxide appeared to be the most promising. Besides, in the complementary experiments (with addition of the Bergmann mixture) all the fractions had to be highly diluted in order to reduce the effect produced by the kidney extract alone.

As pointed out in the work mentioned, however, fullers earth removes a considerable part of the active substance, especially when the absorption is carried out in acetic acid solution, and the active substance may be eluated again. We have investigated, therefore, whether this part might be more accessible to further fractionation.

The experiments gave about the same results for the eluate as for the mother-liquor. None of the methods tried were found to be satisfactory, the active substances being distributed on the various fractions without any distinct separation. This may have been due to unservieeability of the raw material and the methods used, but to a large extent it may also be due to the circumstance that in examining the effect of the cultures it is impossible to obtain even an approximate expression for the quantity of the complementary substances present. On the whole, this circumstance introduces a serious difficulty into investigations of this character. Besides, the fact, that both the mother-liquor and the eluate from the fullers earth treatment give strongly active products, shows that no selective adsorption takes place. Furthermore, both products are fully active without any addition of the Bergmann mixture, so that no fractionation on this basis would be practicable either. This means that separation of the raw material into active and inactive fractions is associated with difficulties which conceivably may be due to the presence of *several* components, each of which exerts a complementary action itself alone or together with other components.

2. Heart Extract.

In order, if possible, to find a less complicated raw material for the production of active solution, some experiments were carried out with extract of ox heart. Heart tissue is known to be rich in enzymes although in itself it is not an enzyme-producing

tissue; especially, in contrast to the kidneys, the heart does not take part in the amino acid metabolism of the organism. Boiled heart extracts were found to have many properties in common with the kidney extracts — as, for instance, thermostability and dialyzability — and yet they prove to be of a different character, as they are inactive by themselves alone and become active merely on addition of the Bergmann mixture.

V-100: 950 g chopped ox heart is boiled for 10 min. with 1.9 l of water and 9.5 ml acetic acid. Left standing till next day for cooling, pressed through gauze, neutralized with 2-n NaOH and filtered. Contains 1.12 mg N/ml. Autoclaving.

In contrast to the kidney extract the heart extract was but very little active when employed by itself alone, but highly active when employed together with the Bergmann mixture. It was also active when used together with a mixture of only cystine, lysine, glutamic acid, tryptophan and arginine. As in the kidney extracts, treatment with fullers earth did not result in any effective separation.

3. Red Blood Cells.

According to SBARSKY (1941) red blood cells contain large amounts of amino acids and even serve as vehicle for the transport of amino acids in the organism. With this in mind we carried out some experiments with extracts of red blood cells. Later, however, USSING (1943) has shown that the increase in the amino acid content of these cells appears to be due exclusively to glutathione. Glutathione (10 mg in 12.5 ml physiological NaCl solution and autoclaved) has no effect on the tissue cultures; indeed it is destroyed by autoclaving and hence it cannot be this substance that constitutes the active component of the boiled extract.

V-88: The blood cells from 4 l oxalated blood are suspended in 5 l physiological NaCl and centrifuged. Then they are suspended in 10 l physiological NaCl and centrifuged. After this they are boiled in 3 l water + 50 ml 1-n HCl. This mixture becomes very thick from the coagulated protein. A little chloroform is added and the mixture is filtered in the ice-box. After 3 days about half a litre of filtrate has passed through the filter and is evaporated a little in a porcelain dish in order to remove the chloroform. Filtration. Contains 0.61 mg N/ml.

Is inactive, but by addition of Bergmann mixture it becomes active. Its action seems identical with the action of the heart extract.

4. Serum Dialysate.

Previous orientating experiments with ultrafiltrates or dialysates of serum (FISCHER 1941 a) showed a slight, but distinct, complementary action which was considerably less than that of normal serum. The explanation may be found in the relatively small amounts of dialysate employed and also in the fact that another method was used for the demonstration of this effect on the culture. We now took up work again with these initial materials, as it seemed reasonable to expect that they might completely supplement the dialyzed media if the dialysis involved no other processes than removal of the dialyzable substances. It was possible, however, that the extracts and solutions used so far and produced by boiling, did not contain the same active substances as fresh serum. The extracts mentioned had a distinct complementary effect, especially on simultaneous addition of the Bergmann mixture, but the growth and appearance of the cultures indicated that these mixtures did not contain quite the same substances as the genuine media, and that they were not able fully to replace the latter. It seemed conceivable, therefore, that the active substances in serum might be of a different nature. In contrast to the substances in the boiled extracts, especially kidney extract, the serum components were unable to stand boiling and autoclaving, — or only to a slight extent. Furthermore, we found that it was necessary to employ far greater amounts of the serum dialysate than we had previously employed of boiled extracts or of dialysate and ultrafiltrate. Only by using an amount of dialysate corresponding to replacement of the total liquid phase in the culture with serum could the dialyzed media be fully restored. This shows that normal serum or plasma does not contain a particularly large surplus of the dialyzable components required.

This is quite in agreement with previous experiments (FISCHER 1941 a; FISCHER and ASTRUP 1942) which show that even though small amounts of normal serum on addition to the dialyzed media are able to prevent the cells from perishing, more than one-half of the mixture must consist of normal serum if the growth is to be normal. With corresponding large amounts of dialysate, how-

ever, cultures are obtained which cannot be distinguished from the normal, neither in growth nor in appearance, and which are superior to the cultures obtained with the best boiled kidney extracts. The cells are vigorous, clear and growing rapidly, whereas the boiled kidney extracts gave cultures with a fairly large area of growth, it is true, but looking very "thin" (thin and slender cells).

V-122: 400 ml ox serum (obtained by centrifuging defibrinated blood) is dialyzed for 3 days at 0° against 400 ml distilled water with addition of a little chloroform and toluene. The dialysate is evaporated in vacuo at a bath temperature below 40° till about 50 ml has passed over, for removal of the chloroform and toluene. A little sediment is removed by filtration.

V-122.1: A sample of the dialysate is autoclaved.

V-122.2: A sample of the dialysate is made slightly acid to lithmus paper by means of 1-n HCl, and then autoclaved.

V-122.3: A sample of the dialysate is sterilized by filtering through a Seitz filter.

Tested on tissue cultures the two samples sterilized by heating were found to be rather inactive, but the sample filtered is active. Even though such autoclaved samples of dialysate are not always completely inactive, the experiment shows that serum components differ from the substances present in kidney and heart extracts. The latter rather improved on autoclaving, but of course, this may be due to the inhibitory influence exerted by the foreign proteins present before their denaturation and removal. Previously, a dialysate of fresh, un-boiled extract of ox kidney had been found to be inactive after sterile filtration; and this indicates that the thermostable, dialyzable components are set free only by the boiling and that only a very slight amount of the thermolabile elements are present in such an extract. Thus there appears to be a difference in the nature of the two types of active substances. Hence it may be that their effects on the tissue culture are not identical, even though they look somewhat alike. This question will be dealt with further in the following discussion of the experimental results.

Further experiments showed that the active substances do not very well stand evaporation of the alkaline dialysate and that addition of dilute hydrochloric acid to a permanent weakly acid reaction, or aeration with carbon dioxide during the evaporation in vacuo give more active solutions.

V-124: 250 ml ox serum is dialyzed as above, the dialysate is evaporated in vacuo to about $^1/_5$ volume — at a bath temperature of < 45° — under continual aeration with CO_2.

V-124.1: 10 ml of V-124 is diluted to 40 ml with distilled water. Neutralization with 1 drop of 2-n NaOH. Sterile filtration.

V-124.2: The remainder of V-124, 45 ml, is mixed with 3 spatulas of Franconite and 1-n HCl to a weakly acid reaction on lithmus paper (20 drops). After standing for 10 min., filtration. The filtrate is diluted with 3 volumes of distilled water. Neutralization and sterile filtration.

V-124.3: The Franconite sediment from V-124.2 is washed once with water, then suspended in 30 ml water and neutralized. Then filtered, and 15 ml of the filtrate is diluted to 50 ml. Sterile filtration.

V-124.1 is quite active on cultures of osteoblasts as well as on heart fibroblasts, and V-124.2 seems to be almost as effective, whereas V-124.3 is only slightly effective on osteoblasts and practically ineffective on heart fibroblasts. The franconite treatment appears not to have removed any significant amount of the active substances.

5. Bottom Yeast.

According to the above-mentioned experiences with serum dialysates, it is not sufficient merely to look for thermostable components, it is necessary also to look for thermolabile substances, and it is even to be expected that in their nature and mode of action these substances will be more in conformity with the active components present in serum. On the other hand, it is very impractical to use serum dialysates as the raw material for further investigation, since it is difficult to obtain it in large amounts, and evidently it does not contain the active substances in any particularly high concentration. Nor do the raw materials previously employed for the production of boiled extracts seem suitable. According to previous experiments fresh kidney extracts do not contain any considerable amount of the thermolabile dialyzable components, which presumably circulate with the blood and are taken up and retained by the cells in the various tissues of the organism.

Previously, in a single experiment, Lebedew juice from bottom yeast showed some effect on the tissue cultures. Pronounced liquefaction of the plasma clot took place, however, especially in the immediate vicinity of the tissue cells, so that the culture soon perished. Presumably this liquefaction is due to proteolytic enzymes present in the Lebedew juice. A boiled dialysate of the

Lebedew juice was almost entirely inactive. Our working hypothesis at that time did not suggest experiments with a non-boiled dialysate. But the possibility still remained that the effect might be due to thermolabile components, and that it would become more conspicuous after removal of the contents of foreign proteins; furthermore, it seemed reasonable to assume that the liquefaction of the plasma would cease on removal of the enzymes. It seemed natural, therefore, to take up these experiments again for further study.

V-129: 40 g of the same dry yeast as that employed in the preliminary experiments, and which had been kept in the laboratory for about $2^1/_2$ years, are mixed with 120 ml water that has been heated to 35°; the mixture is placed on water-bath at 35—40° for 3 hours with frequent stirring. On centrifuging it yields 40 ml extract. This is dialyzed for 2 days against 60 ml distilled water at 0°, with addition of a little chloroform and toluene. The chloroform and toluene are removed from the dialysate by distillation on water-bath (< 45°) in vacuo under aeration with CO_2. After dilution with distilled water to make 40 ml, and addition of 4 ml of ten times concentrated Ringer solution, it is neutralized with 2-n NaOH. Sterile filtration.

This solution was active on osteoblasts and heart fibroblasts. The activity is reduced by autoclaving but may again be restored somewhat by addition of the Bergmann mixture, which also seems to promote the action of the non-autoclaved extract. In nearly all the following tissue culture experiments, therefore, the various solutions have been tried out with an addition of the Bergmann mixture (designated as +B).

The experiments show clearly that active substances are present in the sterile filtered dialysate from the Lebedew juice. Hence the nature of these substances is investigated further with dry yeast produced from CARLSBERG bottom yeast as raw material.

Dry Yeast from Bottom Yeast: About 3 l fresh bottom yeast is suspended in 10 l tapwater and poured through gauze. The suspension is centrifuged and suspended again in 10 l water, then centrifuged once more. Drying at 30—40°, and grinding to a fine powder in a ball-mill.

V-133.2: 40 g dry yeast powder is suspended in 200 ml water heated to 35°; this suspension is kept at 35—40° for 2 hours with frequent stirring, then centrifuged. Volume 110 ml. Dialysis against 165 ml water with addition of a little toluene for 2 days. Volume of dialysate 140 ml. Contains 2.51 mg N/ml.

V-133.21: 130 ml dialysate (V-133.2). is mixed with two volumes 96 % alcohol. This gives a small amount of flocculär sediment and the

mixture filters very slowly. The sediment is dissolved in 100 ml water, filtered and evaporated in vacuo for removal of the alcohol and toluene. Neutralization, dilution with water to 100 ml and addition of 10 ml 10 × Ringer. Contains 0.13 mg N/ml.

V-133.22: From the mother-liquor of V-133.21 the alcohol is removed by evaporation in vacuo. Neutralization and dilution to 100 ml. Contains 2.90 mg N/ml.

While the original dialysate (V-133.2) was rather active alone and even more active +B., the precipitate was practically inactive (+B). This experiment shows that under such conditions the active substances are not precipitated by alcohol. Still, the active substances are apparently not soluble in pure alcohol. On evaporation in vacuo to dryness of the active fraction V-133.22, and treatment with absolute alcohol at 60—70°, some nitrogen-containing substances are extracted, but they prove inactive (+B.). The remnant, on the other hand, is active (+B.). Additional experiments with alcohol precipitation of the dialysates (also after concentration in vacuo) show that the active substances are soluble even in rather strong alcoholic solution. This implies the possibility that the proteins and other substances of high molecular weight present in the extracts may be removed by precipitation with alcohol instead of dialysis — something that would make the initial material far more easily accessible. In subsequent experiments it is found that the yeast extract may be precipitated with 3 volumes alcohol and after filtration and removal of the alcohol by distillation in vacuo yield solutions which in character appear perfectly to correspond to the dialysates.

The stability of the substances derived from the bottom yeast is in investigated further.

V-134.1: 100 ml yeast extract (like V-133.2) is dialyzed against 150 ml water with addition of a little chloroform. The chloroform is removed by evaporation in vacuo. Dilution with water to 100 ml.

V-134.11: 25 ml of V-134.1 is neutralized and sterilized by filtration.

V-134.12: 25 ml of V-134.1 is neutralized and boiled on a water-bath for 1 hour. Autoclaving (1 hour at 110°).

V-134.13: 25 ml of V-134.1 is made acid to Congo red with 1-n HCl and boiled on a water-bath for 1 hour. Neutralization and autoclaving.

V-134.14: 25 ml of V-134.1 is made strongly basic to lithmus paper and boiled for 1 hour on a water-bath. Neutralization and autoclaving.

The sterile filtered extract (V-134.11) is active both on osteoblasts and fibroblasts (+B.). With the other extracts the growth is at first fairly good, especially on heart fibroblasts, but then the growth ceases. The substances present in these extracts are considerably more sensitive to boiling than those present in the extracts previously employed. In order further to establish the difference, an attempt is made to produce a boiled extract of yeast according to the method used for the production of kidney extracts.

V-139: 25 g dry yeast is boiled for half an hour with 250 ml water + 2 ml acetic acid. Neutralization, filtration and autoclaving.

This extract is rather inactive on the cultures (+B.). An ordinary boiled extract, however, undoubtedly contains several proteins and other substances of high molecular weight. It may be that such substances have an inhibitory effect on the growth of the tissue cultures. Boiled kidney extract yielded a highly active dialysate in previous experiments, and accordingly a corresponding dialysate was prepared from yeast.

V-140: A boiled yeast extract is produced in the same way as in Experiment V-139. Neutralization and filtration leave 110 ml extract which is dialyzed against 400 ml distilled water for 2 days. Evaporation of the dialysate in vacuo to about 100 ml. Autoclaving.

This dialysate has no effect on the tissue cells. Thus there is a rather distinct difference between the active substances in the boiled extracts tested before and the active substances present in the bottom yeast.

In order to avoid the dialysis the extracts are produced as follows:

V-143: 95 ml yeast extract is produced in the usual way and mixed with 3 vol. 96 % alcohol. The mixture is left standing a couple of days. After filtration, the alcohol is removed by distillation in vacuo. Neutralization and dilution to 95 ml. Sterile filtration.

This extract is active and appears to be just as effective as a dialysate.

6. Baker's Yeast.

After having established the presence of accessory substances in Lebedew juice of bottom yeast and demonstrated that these substances showed a far greater resemblance to the serum com-

ponents than the substances previously investigated, similar experiments were made with baker's yeast, as this raw material was more easily obtained. We used baker's yeast from "Dansk Gær-central" and found that the extracts from this product appeared in every respect to correspond to the extracts from the bottom yeast. On this account the baker's yeast was employed in several additional experiments. Only slight differences in the properties of Lebedew juice produced from bottom yeast and from baker's yeast were found by LIPMANN (1938).

V-131: 250 g baker's yeast is dried at 35°, after being broken up into tiny pieces. After two days' drying it is ground in a ball-mill. Weight 57 g.

A dialysate is active both on osteoblasts and heart fibroblasts (+B.) and appears just as active as the corresponding dialysate from bottom yeast.

V-154.1: From 40 g dried baker's yeast an extract is prepared like that obtained from bottom yeast, cf. V-133.2. This yields 125 ml of extract. Addition of 375 ml 96 % alcohol. After standing till the following day, filtration and evaporation in vacuo. Neutralization and filtration. The filtrate is diluted to 125 ml with distilled water. Contains 1.79 mg N/ml.

This extract was found to be very active on the tissue cultures (+B.).

V-154.2: The remainder of V-154.1, *i. e.*, 100 ml, is mixed with 25 ml saturated $HgCl_2$ solution and left standing till the next day. Filtration, The filtrate is treated with H_2S. Filtered and evaporated in vacuo; then diluted to 100 ml. 25 ml is with-drawn and neutralized. Contains 1.14 mg ml.

Most of the nitrogen was not precipitated by the mercuric chloride. The extract is fairly active on the cultures (+B.) almost as active as the initial material V-154.1.

V-154.3: 70 ml of V-154,2 are neutralized to Congo paper with 2-n NaOH. Addition of 3.5 g. Franconite in small portions with stirring. Filtration after 15 min. Neutralization and sterile filtration. Contains 1.08 mg N/ml.

Not much nitrogen was removed by this treatment, and the extract seemed just as active on cultures (+B.) as V-154.1. Only very little nitrogen could be eluated from the Franconite (0.04 mg N/ml), and the solution was inactive.

Additional experiments showed that the solutions treated with mercuric chloride and Franconite undergo no change when left standing at 0° for 2 days after addition of dilute NaOH to strong reaction with phenolphtalein (Exp. 156.1).

Under these conditions then, the active components may very well withstand a rather strong alkaline reaction. Ether extraction removed no active substances from the extract, neither from acid nor alkaline solution, and the top layer of ether contained only a small amount of nitrogen or none at all (V-156 and V-157). Autoclaving was injurious to the action of the extract, even though it did not make the extract completely inactive (+B.) (V-157). Boiling of a solution that was acid to Congo or basic to phenolphtalein destroyed the activity of the extract almost completely (V-158, V-161, V-191).

Further experiments were then carried out with baker's yeast that had been dried rapidly at about 70°, yielding a light coloured product.

Of this finely pulverized product 100 g are treated for 1—2 hours with 500 ml water at 35—40° and centrifuged. The extract (as a rule 325—350 ml) is precipitated with 3 vol. 96 % alcohol and filtered after standing for 2—3 days.

V-179.1: An alcohol-treated extract of 100 g yeast is evaporated *in vacuo* till all the alcohol is gone (bath < 50°). Dilution to 330 ml. Neutralization with 2-n NaOH with neutral lithmus paper as indicator. Filtration and sterilization. Contains 2.60 mg N/ml. The extract is active on cultures, both on osteoblasts and heart fibroblasts (+ B.).

V-179.2: 230 ml of V-179,1 are treated with 11.5 g. Franconite. Filtration (volume 210 ml). Contains 2.20 mg N/ml. As usual, not much nitrogen has been removed, and the activity of the extract is good, like that of V-179.1. (+ B.).

V-179.3: 150 ml of V-179,2 is acidified with HCl to pH = 5.8. Treatment with 1.5 g active carbon, and filtration. Contains 1.53 mg N/ml. Evidently the carbon treatment removes somewhat more nitrogen than does Franconite. A sample is neutralized. It was very active on the cultures, like that of V-179.2 (+ B.), so that no active substance appears to have been removed.

V-179.4: 75 ml of V-179.3 is mixed with an additional 0.75 g carbon, and filtered. Nitrogen is not removed and the activity is the same as before.

V-179.5: 45 ml of V-179.3 is acidified with 1-n HCl to pH = 3.0 and then treated with 2 g carbon. Filtration, neutralization and sterile filtration. Contains 0.96 mg N/ml. At this pH somewhat more nitrogen has been removed, but very large amounts are still left in the extract.

Thus more than one-half of the amount of nitrogen present in the yeast extract is found in a form that is adsorbed by carbon only with difficulty or not at all. The activity of the last extract is still good, though inferior to that of the raw material. Hence the active components appear chiefly to be found among the markedly hydrophilic and poorly adsorbable substances.

Also larger amounts of active carbon fail to remove the active components of the extract (V-181: after Franconite treatment the extract contains 2.13 mg N/ml. After additional treatment with 2 g carbon per 100 ml, the extract contains 1.47 mg N/ml. No decrease in the activity (+ B.). There is no particular difference in the amount of nitrogen removed by the carbon treatment between pH 3 and 7 (V-185).

Evaporation of the extract till it begins getting viscous and precipitation with a surplus of alcohol (5—10 vol.) give two fractions which, according to the circumstances, differ in their activity and nitrogen content. If the concentrate is made strongly acid to Congo before the precipitation, the alcoholic solution will contain nearly all the nitrogen (V-165.2: 1.30 mg N/ml), while the precipitate contains but very little nitrogen (V-165.3: 0.13 mg N/ml). The active components have remained in the solution while the nitrogen-poor precipitate is inactive.

A less acid reaction (slightly acid to Congo) gives less nitrogen in the solution, more in the precipitate, and the active components are distributed more evenly on the two fractions (V-168.2: 0.96 mg N/ml; and V-168,3: 0.35 mg N/ml).

On addition of acetic acid to the concentrated extract (5 ml glacial acetic acid to a concentrate of 200 ml extract) and precipitation with 96 % alcohol (100 ml), the result is still less nitrogen in the solution and more in the precipitate: V-181.3 (precipitate): 0.60 mg N/ml; V-181.4 (filtrate): 0.82 mg N/ml. At the same time, the solution has now become less active than the precipitate.

The active components appear to be more easily soluble in the acid alcohol than in the more neutral alcohol. The various studies on the properties of the active components and the experiments on their production in more purified form might possibly indicate the presence of a single substance or of a few closely related substances.

7. Barley Malt.

Yeast extracts may contain considerable amounts of many different substances which possibly are of significance to the growth, and this may cause difficulties in the further purification and eventual isolation of the substances in which we are interested. We have tried, therefore, to see whether we might not find the

same substances in extracts of germinating barley, which is very rich in enzymes and contains substances that are of great importance, for instance, to the growth of yeast.

V-176: Green malt on the 5' day of sprouting, from the *Carlsbergs Breweries*, is dried at 50° and ground to a fine powder.

V-176.1: 40 g dried pulverized green malt (V-176) is stirred up in 100 ml water (35°) for about 2 hours. Centrifuging gives 65 ml extract. Dialysis against 200 ml distilled water for 2 days at 0° with addition of a little chloroform. Evaporation of the dialysate in vacuo for removal of the chloroform. Neutralization. Dilution to 50 ml and sterile filtration. Contains 1.14 mg N/ml.

This extract was active (+B.) both on osteoblasts and heart fibroblasts and appeared in its action to be identical with the yeast extract. Thus malt extracts too contain substances which may replace the factors removed from the dialyzed media that are necessary to the growth of the tissue cells.

V-183.1: 200 g dried green malt powder is stirred with 500 ml. water (35—40°) for 1—2 hours. Centrifuging gives 200 ml extract, which is mixed with 600 ml 96 % alcohol and left standing for a couple of days. After filtration the alcohol is removed by distillation in vacuo, and the extract is diluted to 200 ml. Addition of 10 g. Franconite. Filtration. Neutralization and sterile filtration. Contains 0.92 mg N/ml.

This extract contained less nitrogen than the corresponding yeast extracts; yet it appeared to be rather a little more active on the cultures (+B.). The alcohol precipitation and the Franconite treatment have the same effect on this extract as on the yeast extract. Also treatment of this extract with carbon removed part of the nitrogen (just as in the case of the yeast extract) without any essential reduction in the amount of active substance.

Then experiments were carried out with dried Pilsner malt from the *Carlsberg Breweries* as raw material. This malt, which was dried at about 90°, proved just as active as the green malt dried at a lower temperature.

V-186.1: 200 g pulverized Pilsner malt is stirred for 1 hour with 500 ml water at 35—40°. Centrifuging gives 290 ml extract, which is precipitated with 3 vol. 96 % alcohol and left standing for a couple of days. Filtration. Distillation in vacuo to remove the alcohol from the extract which is diluted to 290 ml. Addition of 14.5 g. Franconite and filtration after standing for a short time. Contains 0.73 mg N/ml which is considerably less than the nitrogen content of the yeast extract at the same stage — about one-half.

This extract was very active on the cultures (+B.). The malt extract thus appears to be an excellent material for further investigation, being easily accessible, and apparently containing the active substances in a purer state than the yeast extract. Hence this malt extract has been employed as the raw material for further experimental studies which will be described in a subsequent paper.

C. Discussion.

Correlation of the above-mentioned experimental results with our previous findings raises various problems. A few of these have already been mentioned (see FISCHER 1942 b and ASTRUP 1943), but the present, more extensive studies have suggested some considerations which deviate from our previous working hypotheses.

The dialysis of the media employed for the tissue culture was aimed originally at removing the amino acids present, as the amino acid contents of the blood were assumed to serve as a source of nitrogen for the tissue cells. It was not possible, however, to compensate the dialyzed media by addition of the amino acid mixture found by ROSE and collaborators (ROSE 1938) to be necessary to and sufficient for an organism, namely, the following acids:

l (+)-valine, (0.7 %)
l-(—)-leucine, (0.9 %)
l-(+)-isoleucine (0.5 %)
l (+)-lysine, (1.0 %)
l (—)-threonine [d(—)-threonine (ROSE)], (0.6 %)
d, l-tryptophan, (0.2 %)
d, l-histidine, (0.4 %)
d, l-phenylalanine, (0.7 %)
d, l-methionine, (0.6 %)
l (+)-arginine, (0.2 %)

ROSE originally performed his experiments on rats, and arginine was found necessary to the growth only, not to the maintenance of the organism. In principle, subsequent experiments have confirmed the original results (see, for instance, WOLF and CORLEY 1938). Dogs react like rats, but other organisms may require some slight change in the amino acid composition. Chicks consume more arginine and require a supply of glycine (ROSE and RICE 1939), whereas man is able to do without histidine (ROSE, HAINES, JOHNSON and WARNER 1943).

From these few amino acids, then, it is possible for a *whole organism* to prepare all the other amino acids essential to the building up of the tissues. A conspicuous feature of ROSE's list of amino acid requirement is that it includes *all the known, naturally occurring, amino acids in which a methyl group occurs outside the straight carbon chain,* namely, the following four acids: *Leucine* (α-amino-γ-methyl-n-valerie acid), *isoleucine* (α-amino-β-methyl-n-valerie acid), *valine* (α-amino-β-methyl-n-butyrie acid) and *methionine* (α-amino-γ-metylthiol-n-butyrie acid). This means that the organism is unable to introduce a methyl group in a side chain, whereas it is able to decompose it and in this way build up other amino acids essential to it, so that, for instance the sulphur-containing amino acid cystine may be formed from methionine, but not vice versa (see TARVER and SCHMIDT 1939).

Now *the individual tissue cells* in the organism are found to behave quite differently from a whole organism. From our previous experiments it is evident that the tissue cells are not able themselves to utilize the dialysed serum and plasma proteins as a source of nitrogen for their nutrition. Nor does the amino acid mixture given by ROSE enable the cells to live and grow on the dialyzed media. The individual tissue cells require sources of nitrogen that are quite different from those required by the organism as a whole, and they are not able to utilize the acids given by ROSE. In the organism the Rose mixture of amino acids serves as raw material for the production of substances which may be utilized by the individual cells in the organism. Hence certain organs, presumably the liver, must contain cells that are able from the synthetic diet given by ROSE to form the nitrogen-containing nutrients required by the other cells of the organism.

Now the question arises: Which substances are so essential to the individual tissue cells that their absence means the death of the cell?

Primarily numerous other amino acids have been tried out in this respect but without satisfactory result. Glycine appears ineffective, and the same applies to all the other acids tried except for cystine, which showed a slight effect. Altogether a number of amino acids have been tried without the cells being able to live and grow as on non-dialyzed media. (Several of the amino acids were kindly placed at our disposal by HOFFMANN-LA ROCHE & Co., Basel).

Some effect was obtained with amino acid mixtures resulting from decomposition of proteins by acids or enzymes, but this was due almost exclusively to the almost insoluble cystine-containing fraction. The amino acid mixture which was found most effective had the composition given by BERGMANN and NIEMANN (1936) for fibrin. Here, too, cystine was the decisive element. Without cystine the rest of the mixture was ineffective and the same was the case when d-cystine was substituted for l-cystine. Among the rest of the amino acids most of them could be omitted without affecting the action, and only lysine and glutamic acid increased the effect of cystine to some degree. The presence of methionine in the mixture did not compensate for an absence of cystine. Denatured or hydrolyzed proteins were able, apparently, in part to replace cystine, even though the products were prepared from proteins which had been dialyzed beforehand.

From these results it was evident that the amino acid and protein metabolism of a tissue culture could not be consistent with that of a whole organism as established by ROSE'S *investigations.* It would be necessary, therefore, to look elsewhere in order to explain the assimilation by the individual cells of the nitrogen-containing elements required for the building-up of the cell, as these elements had to be made available in the organism in some way other than by the supply of an amino acid mixture of simple composition.

The organism must be able, from the amino acid mixture supplied, to provide other substances absolutely necessary to the vital functions of the individual cell.

In this connection, one would naturally remember the transamination process discovered by BRAUNSTEIN and KRITZMANN (1937). In this process, in the presence of the enzyme transaminase (or aminopherase), glutamic acid serves in transferring an amino group to a ketonic acid (pyruvic acid). The organism is able in this manner to produce other amino acids required by it. This agrees very well with our observation that the presence of glutamic acid increases the effect of cystine on the tissue cells. Yet, according to ROSE, glutamic acid is not an amino acid required by the organism and thus it has to be formed by the organism itself. Not all amino acids are equally able to take part in this transamination process (COHEN 1940), and lysine does not take part in it at all, which explains very well why this acid is found among the essential amino acids of ROSE, and our finding that it promotes the effect of cystine on the tissue cultures. As yet, however, we have no adequate explanation as to why cystine occupies a keystone position.

The transamination process appears not to be a sufficient basis for the supply of nitrogen-containing nutrients to the cells — not only because

all amino acids do not take part in the process, but also because even the most variegated mixtures of up to 20 amino acids have failed to produce normal growth of the tissue cells. Therefore the important point probably is not the question of the supply of a more or less sufficient amino acid mixture, but of more specific substances, possibly of an entirely different type. In the intermediate nitrogen metabolism the plasma proteins have been found to play a considerable rôle. This has been established especially through investigations carried out by WHIPPLE et al. and SCHOENHEIMER et al.

WHIPPLE has shown (see MADDEN and WHIPPLE 1940) that dogs may be kept in nitrogen balance by intravenous administration of plasma proteins, which means that these proteins may be utilized directly in the intermediate protein metabolism and need not first be broken down into amino acids in the digestive tract. WHIPPLE assumes, therefore, that the plasma proteins may be utilized directly by the individual cell in the organism without preliminary degradation. This is not in accordance with our investigations, however, which show that the cells are *not* able to utilize the dialyzed plasma proteins. But perhaps the conditions in a whole organism are different in this respect — a question to which we shall return later.

WHIPPLE found that cystine is of particular significance to the formation of the plasma proteins and that in this respect it cannot be replaced fully by methionine — which appears to be in agreement with our findings concerning the significance of cystine. He arrived at the result that, in contrast to the assumption of classical physiology, the plasma proteins do not remain in a static unchangeable state, having merely physico-chemical and immunological functions to perform in the organism, but that a very rapid exchange takes place between the proteins of the plasma and the tissue, thus establishing a dynamic balance. On this very account, the physiological function of the plasma proteins is assumed to be largely that of playing a rôle in the intermediate protein metabolism.

The views advanced by WHIPPLE have been confirmed and amplified most beautifully by SCHOENHEIMER et al. (cf. SCHOENHEIMER and RITTENBERG 1940 and SCHOENHEIMER and RATNER 1941) in studies on amino acids and proteins containing isotopes. These authors found that in the living organism a rapid exchange takes place between the various proteins, and that particularly the plasma proteins take part in it. On the whole, the proteins of the organism exchange much more intensively with the nutrients supplied than was imagined according to the classical physiology, and the living tissue is in constant activity in order to maintain its structure. There is an uninterrupted process of desamination and reamination, and the dicarbonic acids play a special rôle, participating especially in the nitrogen exchange. Lysine, on the other hand, does not take part in the transamination (WEISSMAN and SCHOENHEIMER 1941). Also the carbon skeleton of an amino acid in a protein may be exchanged with the carbon skeleton of an amino acid supplied. This is evident especially from studies on l(—)-leucine reported by SCHOENHEIMER, RATNER and RITTENBERG (1939)

but the mechanism of this process is still obscure. Thus the intermediate amino acid metabolism is quite extraordinarily intensive in the living organism.

All the investigations mentioned (ROSE, WHIPPLE and SCHOENHEIMER) concerned the amino acid absorption and protein metabolism *in a whole, living, organism*, and they were not able to say anything as to how *the individual cell* in the organism absorbs and synthesizes its proteins. They have shown, however, that the plasma proteins may play a special rôle in this metabolism. Tissue culture experiments ought to be suitable for the solution of this problem, but as late as in 1936 ALCOCK said: "The tissue culture, the only case of protein synthesis under any measure of control, carries out its work as secretly as the whole animal, and has so far yielded no information".

The difficulties one encounters in such work are evident already from a previous work (FISCHER 1941), and here it will suffice to mention that two tissue factors were found to be required for the *growth* of the cells in the plasma medium. One is a non-dialyzable protein-like, very thermolabile growth substance which we have called "embryonin" (FISCHER 1941 b; FISCHER and ASTRUP 1943). Only a very slight amount of this substance is present in plasma, and usually it is added to the tissue culture in the form of an embryonic extract.

While "embryonin" appears not to be necessary to the maintenance of the life of the cells but merely to their multiplication, the plasma contains some dialyzable, rather thermolabile factors which are necessary also if the cells are to be kept living. Normally these substances are present in the plasma in sufficient amounts to allow embryonin to exert its growth-promoting effect freely. But if they are removed from the plasma by dialysis the cells will soon perish, irrespective of the presence of salts, glucose and embryonin.

In view of what has just been said about the active part played by the plasma proteins in the intermediate metabolism, this observation is surprising, as the cells have an excess of plasma protein at their disposal. But evidently the cells are not able to utilize these substances under the experimental conditions.

Our problem may therefore be divided into two questions:

1. Are the plasma proteins of no direct significance to the nutrition of the cells, the cells utilizing the dialyzable factors of low molecular weight in the plasma?

2. Or do the dialyzable accessory substances of low molecular weight enable the cells to utilize the plasma proteins?

1.

It would be natural to assume that the cells utilize the amino acids of the blood and that it is the absence of these acids in the dialyzed media that prevents the cells from growing. At first

this idea was the guiding principle of our studies. In this way the absorbed nitrogen-containing food might be distributed through the blood stream as amino acids to the individual cells and here utilized in the formation of the cell's own proteins.

As mentioned, however, it has been impossible for us to find an amino acid mixture that was able completely to re-establish the normal nutritional properties after the medium had been dialyzed. Still, some amino acids — especially cystine, but also glutamic acid and lysine — had a pronounced effect in this respect.

This effect was most conspicuous, however, when the medium was dialyzed merely to a moderate degree, whereas it almost failed to appear when the dialysis was more complete. Nor did the addition of various vitamins (vitamins C, B_1 and B_2, pantothenic acid, nicotinic acid) or other growth substances (biotin, mesoinosit, β-alanine), with or without simultaneous addition of the Bergmann amino acid mixture, give any additional increase of the growth in the presence of embryonin (in the form of dialyzed embryonic extract).

As already stated, the plasma proteins appear to play a special rôle in the protein metabolism of the organism; through the capillary network these proteins are indeed made available to all the cells in the organism. All cells exert further a thromboplastic effect and make the fibrinogen of the blood plasma coagulate on their surface. One of us has already advanced the hypothesis that fibrinogen (and fibrin) are of particular importance to the nutrition of the cells (FISCHER 1936). Subsequently we found that this idea had been advanced by NOLF as early as in 1913.

A thin layer of fibrin and possibly other denatured proteins will be found on all cellular surfaces. These proteins may be split by the proteolytic enzymes present in the plasma or on the surface of the cell. The cleavage products thus produced may diffuse into the cell and be utilized by it to build up its own proteins. It may be that the composition of fibrin is particularly suited for nutrition of the cells, and for this reason we tried the amino acid composition given for fibrin by BERGMANN and NIEMANN (1936). According to the findings reported by SCHOENHEIMER, RATNER, RITTENBERG and HEIDELBERGER (1942) there does not seem to be any particular difference in the tendency of the various plasma proteins to exchange amino acids with the organism.

It has to be pointed out, however, that in this respect the different cells may behave individually.

If the amino acids or similar substances of low molecular weight present in the blood serve as elements for the building up of the proteins of the individual cells, there must be a mechanism which regulates the amount of each one of the necessary amino acids present in the blood, in order to prevent a fall below a certain limit. This means that each acid has to be made available, and its amount regulated, independently of the other amino acids. It is obvious that this requires a very intricate mechanism in the organism.

Matters are different, however, if fibrinogen (and possibly other blood proteins) serves as a source of amino acids. The composition of fibinogen, then, may be of such a character that it includes the necessary components in favourable proportions — as already suggested in a previous work (FISCHER (1941 a). In this case only one single mechanism is required to build up and break down the fibrinogen and regulate its concentration. In this way the whole matter becomes very much simplified. As a matter of fact, this principle is not unknown in the organism. In the nutrition of the infant the casein formed by the mammary glands actually serves as the source of amino acids, and it might just as well be replaced by a mixture of the free amino acids, since — unlike the other proteins of the organism — it is only a nutrient and serves no other purpose (enzymatic or structural). It is a far more simple task to regulate the formation and concentration of a single substance of an established constitution than to regulate the amount of each amino acid entering into the composition of this substance.

Accordingly, even though the amino acid content of the blood appears to be constant, it may not be an expression for a real regulatory mechanism, as is the glucose content. On the contrary, the presence of the amino acids may be interpreted as an image of the processes of decomposition and synthesis taking place incessantly in the organism, being thus an expression for a dynamic balance between these processes, not for a statically maintained state. Naturally the composition of the blood will reflect these processes. As the blood flows past all the cells of the organism, it will also take up some of the cleavage products from the proteolytic processes in the fibrin clots on the surface of the cells even though these substances possibly are formed for the use of

the cell concerned. A part of these substances diffuses into the cell, but another part diffuses out of the clot and enters the blood stream. The amino acids thus taken up by the blood may in the liver be resynthesized into plasma proteins — together with the amino acids supplied to the liver by the nutritional elements absorbed from the digestive tract.

2.

So far, however, it has been impossible to find an amino acid mixture, on which the cells grow satisfactory in the dialyzed media, and the amino acids examined have thus not been able alone to serve as adequate nutrients for the cells. One might imagine that the splitting of the proteins into substances of low molecular weight does not proceed to the amino acid stage, and that various polypeptides serve as food for the individual cells. Indeed, more recent investigations — *e. g.*, GODFRIED (1939) — have shown that the circulating blood also contains small amounts of polypeptides. Naturally, this polypeptide content may be looked upon in the same way as the amino acid content, namely as derived from enzymatic processes on the surface of the cells.

Experiments with solutions produced by treating dialyzed proteins with pepsin and erepsin (FISCHER 1942 a) show that homologous proteins which are not quite broken down have a greater effect than the smaller cleavage products. Also extracts obtained by boiling of kidney yield a considerably better growth (FISCHER and ASTRUP (1942). Fractionation experiments with the last-mentioned product gave unsatisfactory results, however, as it was found that the activity was not associated with certain fractions alone but was distributed throughout all the products. The experiments with kidney extracts were further complicated by the circumstance, that the cultures, after growing on the extracts + the amino acid mixture for some length of time, proceeded to grow even if one of these components was removed from the mixture. While these findings cannot be explained for the present, they remind of analogous experiences — for instance, in studies on the growth substances of yeast, especially pantothenic acid (WILLIAMS, EAKIN and SNELL 1940). As shown by the present studies, boiled extracts from other organs (heart, blood corpuscles) failed likewise to give satisfactory results with regard to fractionation; nor did their effect fully correspond to that of the genuine media.

3.

As we did not fully succeed in composing a nutrient mixture for the cells to replace the substances removed by dialysis, the problem had to be approached in some other way. Consequently the active substances present in serum dialysate were investigated more thoroughly. These substances were found to be far more sensitive, especially more thermolabile, than the substances present in the boiled extract, and thus it was ascertained at once that they must be of a nature quite different from that of the substances investigated so far.

Further studies established with certainty that they were different from the previous boiled extracts; they also showed that substances with corresponding properties could not be isolated from fresh kidneys. But even in plasma and serum the amounts of thermolabile substances were rather small. On the other hand, substances with the same properties were found in abundance in yeast and malt, and more thorough investigations showed more and more distinctly the difference between the new substances and the components of the boiled extracts. The new substances are very hydrophilic and are adsorbed only with difficulty by fullers earth and active carbon. In many respects their properties remind one of the so-called "filtrate factor" which subsequently proved to be pantothenic acid. But, as pantothenic acid (obligingly furnished us by Dr. NIELS NIELSEN, Copenhagen) is inactive, the properties of the extracts cannot be due to this substance.

The occurrence of substances essential to the maintenance and growth of animal tissue cells demonstrated in products from yeast and barley malt is significant and indicates a universal distribution and importance of the substances concerned. One might imagine that some of the known co-factors present in yeast extracts were responsible for the effect, but we have not been able to obtain satisfactory results in experiments with cocarboxylase and cozymase.[1] A few orientating experiments with other plant extracts have also been made. Oat, barley and potato appear to be quite inactive in this respect, while kale was only slightly active and did not give normal cells. So it may be that the active substances are present only in certain cells and only under special conditions, presumably in growing cells.

[1] Our thanks are due to Prof. HANS VON EULER, Stockholm, for his kind assistance.

The presence in yeast of substances promoting the growth of animal cells has so far been mentioned only by HEATON (1926). He used an inadequate technique, and his results require confirmation. We have treated dried yeast with alcohol in order to obtain active substances. But neither extraction with 96 % alcohol at room temperature nor at the boiling point gave active extracts.

Plant extracts (germinating maize) have been investigated by PAULMANN (1937) who reported that his extracts had a growth-promoting effect resembling that of the embryonic extract, embryonin. He added but small amounts of extract, and the substances he obtained are far more likely to be of the type we have investigated, which require the presence of embryonin to exert any growth-promoting effect on which account we always work with addition of an excess of dialyzed embryonic extracts. PAULMANN (1939) has later reported investigations of the chemical properties of the active extracts. In contrast to our substances they appear to be precipitated in 50 % alcoholic solution in the presence of proteins. After drying, however, they dissolve in 50 % alcohol. So the effect of PAULMANN's extracts might perhaps become more pronounced if the extracts were examined on dialyzed media with the aid of our technique.

It may be mentioned that, in addition, HEATON (1926) and MEDAWAR, ROBINSON and ROBINSON (1943) found also growth-*inhibitory* substances in yeast and malt.

In the present work we took it for granted that the active substances in question would supplement the nutritional quality of the medium in the form of a more or less complete amino acid mixture and that it might be a question of a simultaneous effect of several components. As a rule, therefore, we have always checked the action of the extracts by simultaneous addition of the Bergmann mixture to the cultures. Also the findings with boiled extracts of kidney and heart and of the protein hydrolysates indicate that they act as a supplementum to the amino acid mixture added, the cystine content of the mixture apparently being of importance. As a rule, however, the untreated extracts were also quite active in the absence of the Bergmann mixture, whereas it was possibly by fractionation to obtain some products, the action of which was greatly promoted by the Bergmann mixture. These findings, together with the fact that all the solutions obtained by fractionation of the boiled extracts proved to possess some activity, especially in connection with the Bergmann mixture, seem to indicate the presence of several substances, which are able to replace nutrients normally used by the cells.

Apparently, however, the situation is quite different with the incompletely thermostable substances obtained from the serum

dialysates, yeast and malt. The fractionation experiments indicate that we are here dealing with a single substance or with a few closely related substances, since it was possible to preserve the full activity in some of the solutions by the fractionation, whereas the others proved almost completely inactive even in the presence of the Bergmann mixture. The purified extracts, moreover, were found to be fully active in the absence of the Bergmann mixture. Also the universal distribution of the active products indicates that it may consist of a single or a few components, since it is difficult to imagine how a complex nutrient mixture, acting on animal tissue cells, might be so widely distributed in nature and preserve its complexity even after repeated chemical treatment.

Another fact illustrates the difference between the effect of the thermostable, boiled extracts and the thermolabile elements of the serum dialysate, yeast and malt. With the last-mentioned substances it was possible to obtain tissue cultures which in their growth and appearance were equal to normal cells grown in genuine media; moreover, this was true also of the preparations obtained by fractionation. In contrast, it was not possible with the boiled extracts and protein hydrolysates to obtain cells which corresponded morphologically to the normal. Sometimes the area of growth was satisfactory — and this was particularly the case when using kidney extract — but the morphological appearance of the cells always indicated a growth that was not quite normal. The cells were thin and slender and showed marked granulation and pronounced vacuolization.

Differences in the effect on the tissue cultures may thus point to the existence of two different kinds of active extracts. As a matter of fact, there are also two fundamentally different possibilities of inducing a natural growth of the cells on the dialyzed media.

The first of these possibilities consists in finding a nutritional mixture which completely compensates for the mixture taken up by the cells under normal conditions. This is the possibility we have used as a working hypothesis in the past, although it has not yet led to fully satisfactory result.

The second possibility is to enable the cells to utilize the dialyzed plasma proteins as a source of the necessary amino acids, a possibility which has been discussed briefly in a previous paper (FISCHER and ASTRUP 1942).

Here the question is: Do the cells under normal conditions utilize only the dialyzable substances present in the circulating blood, or do the cells themselves produce the required amino acids by splitting the plasma proteins? In the latter case, certain dialyzable substances must be present to enable the cells to perform the required decomposition of the plasma proteins.

Of course, it will be very difficult to find the proper composition of the food for the cells if this should consist exclusively of various elements of low molecular weight which diffuse into the cell and are consumed by it. It is not to be expected that the composition of the food for the individual cells in this respect will be just as simple as that for the whole organism, because the complete animal organism has several means of transforming the food-stuffs, at its disposal which the individual cell is lacking. Indeed our experiments have shown that the diet, which according to ROSE is sufficient for the maintenance of the whole organism and for keeping it growing, is quite inadequate for the individual tissue cells.

The matter is simple, however, if the cells may be able to utilize the plasma proteins, and, as mentioned already, some evidence makes it rather probable that the plasma proteins actually serve as the means of making the required amino acids available to the individual cells. However, tissue culture experiments show, too, that the dialyzed proteins cannot be utilized as such, but that the presence of certain components of low molecular weight normally found in the blood plasma are required. It may be that this requirement involves merely a single component, and the experiments with yeast and malt extracts indicate that we meet with the same substance here. If so, the problem will be limited to that of looking for this component, which evidently serves as activator for certain systems of enzymes.

That this may be the case is, *inter alia*, evident from the fractionation experiments which do not indicate that a mixture of many, widely different nutrients is to be taken into consideration. It must be taken for granted, however, that the amino acid mixture, found by ROSE to be the minimum of amino acids required by a whole animal organism, must be necessary also to the life of the individual cell. But as the amino acids of this mixture are not supplied to the cultures, and the growth nevertheless is normal when yeast and malt extract are added, even when these extracts have been submitted to further purification,

the only possibility would seem to be that the cells are able themselves to get these amino acids by cleavage of the available dialyzed proteins. The substance looked for does not seem to be a supplementary nutrient, but rather a factor necessary to the utilization of the plasma proteins by the cells; and the conditions in the tissue cultures appear here to reflect the conditions in the living organism, where the same mechanism has to be assumed to be at work.

Such a condition is not new, however, SPERRY and RETTGER (1915) having found that pure proteins are not attacked by putrefying bacteria, and that products of low molecular weight must be present to enable the bacteria to attack the proteins.

Summary.

1. In continuation of our studies on the dialyzable substances required for the growth of animal tissue cells, extracts prepared from kidney, heart and blood corpuscles have been investigated. The experiments indicate that the active substances present in these extracts are not of the same character as the substances present in the genuine media, since the former cannot completely replace the latter.

2. Dialysates of blood serum are found to contain active components which cannot stand boiling. Similar substances are found in extracts of yeast and barley malt.

3. While the boiled extracts appear to contain a mixture of various nutrients, the active extracts of yeast and malt appear to contain only one or a few closely-related substances which probably serve as activators for the utilization of the plasma proteins by the cells.

4. These thermolabile accessory growth substances appear to be of a highly hydrophilic character as their adsorption from an aqueous solution is difficult and they are insoluble in pure organic solvents. On the other hand, they are soluble in dilute alcohol. They are not precipitated by mercuric chloride at neutral reaction.

5. The significance of these findings to the understanding of the protein metabolism of the tissue cells is discussed.

The studies here reported have been carried out with the aid of grants from the *National Association for the Control of Cancer*, *King Christian X's Foundation* and *Danmarks tekniske Højskoles Fond for teknisk Kemi.*

References.

ASTRUP, T., Kemisk Maanedsblad 1943. *24.* 133.
BERGMANN, M., and C. NIEMANN, J. Biol. Chem. 1936. *115.* 77.
BRAUNSTEIN, A. E., and M. G. KRITZMANN, Enzymologia 1937. *2.* 129.
COHEN, P. P., J. Biol. Chem. 1940. *136.* 565. 585.
FISCHER, A., Hospitalstidende 1936. *79.* 841.
FISCHER, A., Acta Physiol. Scand. 1941 a. *2.* 143.
FISCHER, A., Ibidem 1941 b. *3.* 54.
FISCHER, A., Ibidem 1942 a. *4.* 207.
FISCHER, A., Naturwissenschaften 1942 b. *30.* 665.
FISCHER, A., and T. ASTRUP, Pflüg. Arch. ges. Physiol. 1942, *245.* 633.
FISCHER, A., and T. ASTRUP, Ibidem 1943. *247.* 34.
GODFRIED, E. G., Biochem. J. 1939. *33.* 955.
HEATON, T. B., J. Path. Bact. 1926. *29.* 293.
LIPMANN, F., C. r. Lab. Carlsberg, Ser. Chim. 1938. *22.* 317.
MADDEN, S. C., and G. H. WHIPPLE, Physiol. Rev. 1940. *20.* 194.
MEDAWAR, P. B., G. M. ROBINSON, and R. ROBINSON, Nature 1943. *151.* 195. (Chem. Zentralbl. 1943 I. 2600.)
NOLF, P., Ergebn. Med. Kinderheilk. 1913. *10.* 334.
PAULMANN, F. K., Arch. exp. Zellforsch. 1937. *20.* 253.
PAULMANN, F. K., Biochem. 1939, *300,* 153.
ROSE, W. C., Physiol. Rev. 1938. *18.* 109.
ROSE, W. C., and E. E. RICE, Science 1939. *90.* 186.
ROSE, W. C., W. J. HAINES, J. E. JOHNSON, and D. T. WARNER, J. Biol. Chem. 1943. *148.* 457. (Chem. Zbl. 1944 I. 23)
SBARSKY, B., Enzymologia 1941. *9.* 302.
SCHOENHEIMER, R., and S. RATNER, Ann. Rev. Biochem. 1941. *10.* 197.
SCHOENHEIMER, R., and D. RITTENBERG, Physiol. Rev. 1940. *20.* 218.
SCHOENHEIMER, R., S. RATNER, and D. RITTENBERG, J. Biol. Chem. 1939. *130.* 703.
SCHOENHEIMER, R., S. RATNER, D. RITTENBERG, and M. HEIDELBERGER, J. Biol. Chem. 1942. *144.* 541. (Chem. Zbl. 1943 I. 1072.)
SPERRY, J. A., and L. F. RETTGER, J. Biol. Chem. 1915. *20.* 445.
TARVER, H., and C. L. A. SCHMIDT, Ibidem 1939. *130.* 67.
USSING, H. H., Acta Physiol. Scand. 1943. *5.* 335.
WEISSMAN, N., and R. SCHOENHEIMER, J. Biol. Chem. 1941. *140.* 779.
WILLIAMS, R. J. W., R. E. EAKIN, and E. E. SNELL, J. Amer. Chem. Soc. 1940. *62.* 1204.
WOLF. P. A., and R. C. CORLEY, Amer. J. Physiol. 1939. *127.* 589.

From the School of Dentistry and the Zoophysiological Laboratory, Copenhagen.

Treatment of Saliva Samples used for Calcium Analysis.

By

BODIL SCHMIDT-NIELSEN.

Received 24 October 1944.

When calcium is determined in an organic fluid, like saliva, questions about the preliminary treatment arise: 1. Must epithelial cells and other débris in the saliva be removed by centrifugation before the analysis? 2. Is it necessary to remove the proteins (by ignition or by precipitation)?

For serum the neeessity of deproteinization has been discussed, and many investigators (see BECKS and WAINWRIGHT 1934, p. 391) have found that the recovery of calcium is satisfaetory without preliminary deproteinization. VAN SLYKE and SENDROY (1929) however have shown that too low results will be obtained (5 to 15 per cent) if the proteins are not removed. They have also shown that the deficit was due to non precipitated calcium. In spite of these results calcium determinations are still most commonly carried out without deproteinization. (e. g. RAPPAPORT 1933, LARSSON and GREENBERG 1938, SOBEL and SOBEL 1939)

Former investigators have treated the saliva in many different ways. As an example SIGNE JONSGAR (1937) has removed the proteins in saliva from adult persons (by ashing) but the saliva from children she centrifuged as a preliminary procedure. The deproteinization has been used by most former investigators. BECKS and WAINWRIGHT (1934) have published an extensive critique of the literature with references to the calcium content of the saliva. From this paper it is seen that among the investigators only one (BECKS 1929) has used preliminary centrifuging, while preliminary deproteinization has been used by several.

BECKS and WAINWRIGHT are of the opinion that both preliminary centrifuging and deproteinization are necessary. They have found higher calcium values when the samples are not centrifuged, and they conclude that the higher results are due to calcium containing "débris" (such as cells or food remnants). About the preliminary centrifuging they say: "This procedure is necessary, despite possible loss of calcium — a loss that should be negligible and of less serious consequence than the high values resulting from presence of débris, etc".

Own Investigations.

On account of the divergence in the opinions, the author has wanted to study the following problems:

1) Is there any difference in the analytical results obtained from fresh saliva and from saliva stored for 24 hours?

2) What is the effect of deproteinization or centrifuging of the saliva before the analysis is carried out?

Mixed saliva (produced by chewing paraffine) has been used for the investigation. The calcium determinations are carried out according to the method described in the following paper. Deproteinization has been carried out with trichloracetic acid. (Investigations have shown that removal of proteins by ashing and precipitation by trichloracetic acid give the same results.)

In fig. 1 the results of the investigations are given. Saliva from three persons, A, B and C has been used. Each figure represents the average of two parallel analyses. The percentage error of a single calcium determination is 0.7.

The preliminary treatment of the saliva samples can be seen from the figure. The saliva can be centrifuged or not centrifuged, deproteinized or not deproteinized, analysed immediately after the secretion or stored for 24 hours at 5°, 25° or 37°.

It will be seen that:

1) The storage has no influence upon the results when the saliva is not centrifuged but deproteinized before the analysis (after the storage).

2) When the saliva has not been deproteinized the results, in nearly all cases, will be a little lower than those from deproteinized samples (from 0 to 4 per cent). This perhaps is due to protein combined calcium.

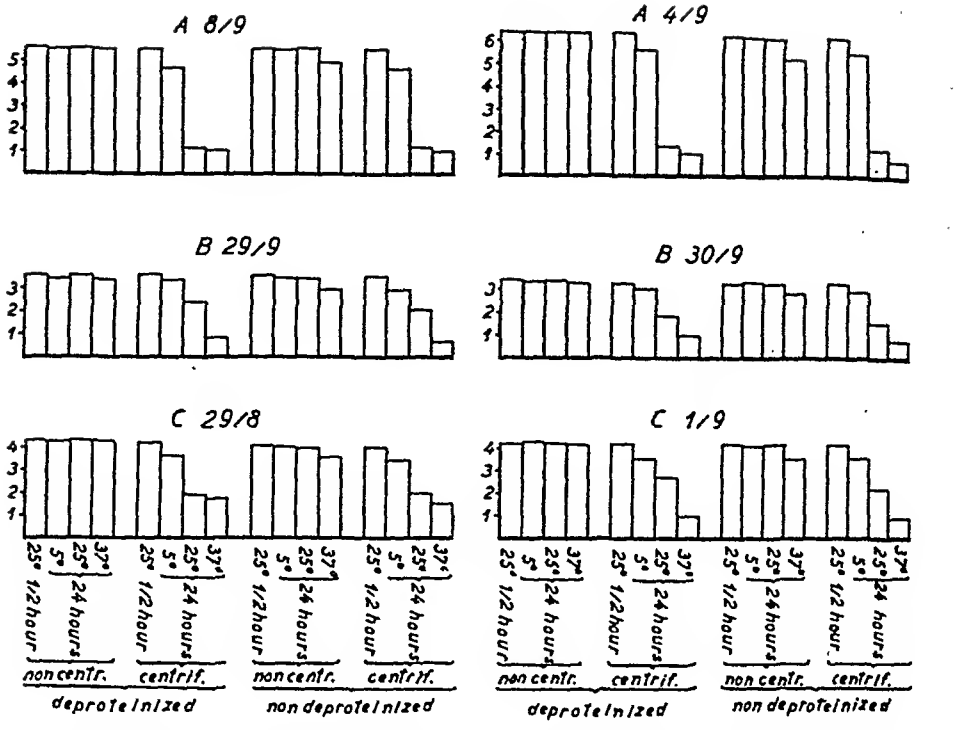


Fig. 1. The influence of different preliminary treatments of saliva samples upon the results of calcium analyses. Samples from three persons, A, B, and C.

Ordinate: mg Ca per 100 ml saliva.

3) When the saliva has been stored the centrifuging has a considerable influence upon the results. If the saliva sample is quite fresh, the centrifuging seems to have no influence. If the saliva has been stored for some time the results will be remarkably lower, and the deficit will increase with increasing tempera-

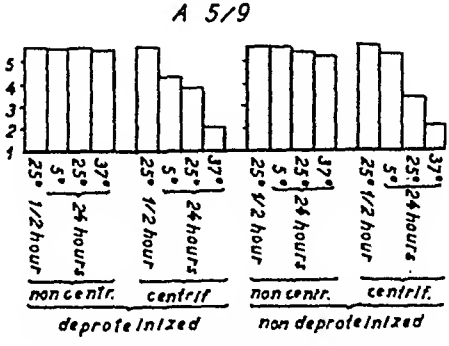


Fig. 2. The influence of different preliminary treatments upon a sample of pure parotid saliva from person A.

Ordinate: mg Ca per 100 ml saliva.

ture. This must be due to a precipitation of calcium during the storage of the saliva.

Fig. 2 gives the results from pure parotid saliva. This sample is taken directly from the parotid duct by means of a saliva separator constructed according to GORE (1938). The saliva taken in this way is quite free from the so-called "débris". The results are very similar to those described above. From this it is evident that the variations that occur by different treatments of the saliva are not produced by the presence of débris.

Conclusion.

The correct preliminary treatment of saliva before calcium analysis is deproteinization.

The saliva must not be centrifuged before the deproteinization.

Storing of the saliva will not influence the results if the samples are thoroughly mixed and deproteinized by trichloracetic acid before the analyses.

Literature.

BECKS, H., Ergebnisse neuerer Speichel-Untersuchungen mit besonderer Berücksichtigung der Gravidität und der Paradentosen. Meusser, Berlin 1929.
BECKS H., and W. W. WAINWRIGHT, J. dent. Res. 1934. *14.* 387.
GORE, J. T. Ibidem, 1938. *17.* 69.
JONSGAR, S., Ibidem 1937. *16.* 173.
LARSON, C. E., and D. M. GREENBERG, J. biol. Chem. 1938. *123.* 199.
RAPPAPORT, F., Klin. Wschr. 1933. *12.* 1774.
SCHMIDT-NIELSEN, B., Acta physiol. Scand. 1945. *9.* 166.
SOBEL, A. E. and B. A. SOBEL, J. biol. Chem. 1939. *129.* 721.
VAN SLYKE, D. D. and J. SENDROY jr., Ibidem 1929. *84.* 217.

From the School of Dentistry and the Zoophysiological Laboratory. Copenhagen.

Microdetermination of Calcium in Saliva and Serum.

By

BODIL SCHMIDT-NIELSEN.

Received 24 October 1944.

The large number of methods for microdetermination of calcium available in the literature shows that this analysis involves many difficulties. The accuracy of most of the methods commonly used is not very satisfactory, and the errors are diffucult to control and can be rather large if the analyses are not carried out with meticulous care.

In nearly all calcium determinations the calcium is precipitated as the very insoluble oxalate. The calcium oxalate can be determined by different procedures viz. gravimetric, titrimetric or gasometric. In microestimations the titrimetric methods are most frequently used. The titration can be either oxidimetric or acidimetric.

The oxidimetric titration of calcium oxalate has gained much favour in actual practice owing to its relative simplicity. HALVERSON and BERGEIM (1917), KRAMER and TISDALL (1921) and many others use a very dilute potassium permanganate solution as oxidizing agent. This method, however, is not very accurate owing partly to the instability of the permanganate solution, and partly to the fact that the endpoint of the titration is not sufficiently well defined.

RAPPAPORT (1933), WILLARD and YOUNG (1928), KATZMAN and JACOBI (1937) and others make use of the more stable ceric sulfate as oxidizing agent, and obtain better reproducible results than by the application of permanganate.

The acidimetric titration has been employed among others by HAMILTON (1925), TREVAN and BAINBRIDGE (1926) and FISKE and ADAMS (1931). The procedure used by these authors has been as follows: The calcium oxalate has been converted by ignition to carbonate, the carbonate dissolved in hydrochloric or phosphoric acid and the titration carried out with an alcali. The advantage of this procedure is that the titration endpoint is well defined; but as in the oxidimetric method the titration solution is not quite stable.

A much better acidimetric principle has been indicated by SOBEL and SKLERSKY (1938). Instead of dissolving the carbonate in a strongly dissociated acid they apply a hot 10 per cent boric acid solution. The titration can then be carried out directly with an acid. Thus the indirect titration, involving the use of the unstable alcali solution is completely avoided.

On account of these desirable features the principle of this method, indicated by SOBEL and SKLERSKY (1938) and modified for microestimations by SOBEL and SOBEL (1939), has been adopted by the author. By the introduction of a number of improvements the accuracy has been increased and the duration of the procedure has been shortened.

Method.

The method is adapted for estimations of calcium in about 0.3 ml of serum or 1 ml of saliva (0.03 to 0.05 mg Ca). If the strength of the hydrochloric acid employed is diminished, smaller amounts of calcium can be determined, but with reduced accuracy.

In the daily routine I have worked with 24 simultaneous analyses, which could be carried out in two times three hours.

a. Reagents.

1. *Trichloracetic acid, 20 per cent.* 10 g of pure trichloracetic acid are dissolved in distilled water and made up to 50 ml. It is not convenient to make more at a time, because it is not completely stable. The solution must be kept in the ice chest.

2. *Ammonium hydroxide, about 1 n.* 68 ml of 25 per cent ammonia are diluted to 1 liter.

3. *Hydrochlorid acid, about 1 n.*

4. *Acetate buffer, pH 4.6, 0.2 n.* Equal parts of 0.2 n sodium acetate and 0.2 n acetic acid are mixed. 0.2 n sodium acetate is prepared by dissolving 27.22 g of sodium acetate ($NaC_2H_3O_2$ $3H_2O$) in water and making up to 1 liter. 0.2 n acetic acid is prepared by diluting 11.3 ml of 99 per cent glacial acetic acid to 1 liter.

5. *Brom cresol green, 0.005 per cent.* 50 mg of brom cresol green are moistened with alcohol and dissolved in 1 liter distilled water.

6. *Ammonium oxalate, saturated.* 6 g of ammonium oxalate are dissolved in 100 ml hot distilled water and allowed to cool.

7. *Ammonium oxalate, 0.5 per cent.* 5 g of ammonium oxalate dissolved in 1 liter distilled water.

8. *Boric acid, 10 per cent.* 10 g of recrystallized boric acid are dissolved in 100 ml distilled water by heating. The solution is always heated just before use, so that all the boric acid is dissolved.

9. *Hydrochloric acid, 0.1 n standard solution.*

b. Apparatus.

Pipettes. Two sorts of pipettes are used in the method: 1. Pipettes of the type developed in the Carlsberg laboratory (Holter, 1943, p. 434—35), but modified to a larger volume (fig. 2). 2. Syringe pipettes (Krogh, 1935).

The accuracy of a 1 ml syringe pipette is about 0.01 per cent, and of a Carlsberg pipette of the same size 0.1 per cent. The syringe pipettes are extraordinarily accurate, and furthermore they are extremely convenient to handle. When the same volume of a reagent is to be added to a number of samples, these pipettes will save much time. On the contrary they are not well suited for pipetting of many different samples, because the dead space of these pipettes claim a thorough cleansing between each sample. In this case the Carlsberg pipettes are more convenient. When the composition of the samples does not differ too much, the washing of the pipettes can be neglected.

The sizes of Carlsberg pipettes used are: about 1 ml, 0.8 ml, 0.4 ml and 0.2 ml. Of syringe pipettes it is convenient to have pipettes in the sizes: 2 ml, 1 ml, 0.5 ml and 0.2 ml.

Special centrifuge tubes in hard glass. In fig. 1 the centrifuge tubes are shown. The wall thickness is 1 mm, the internal diameter about 10 mm and the over-all length about 80 mm. The

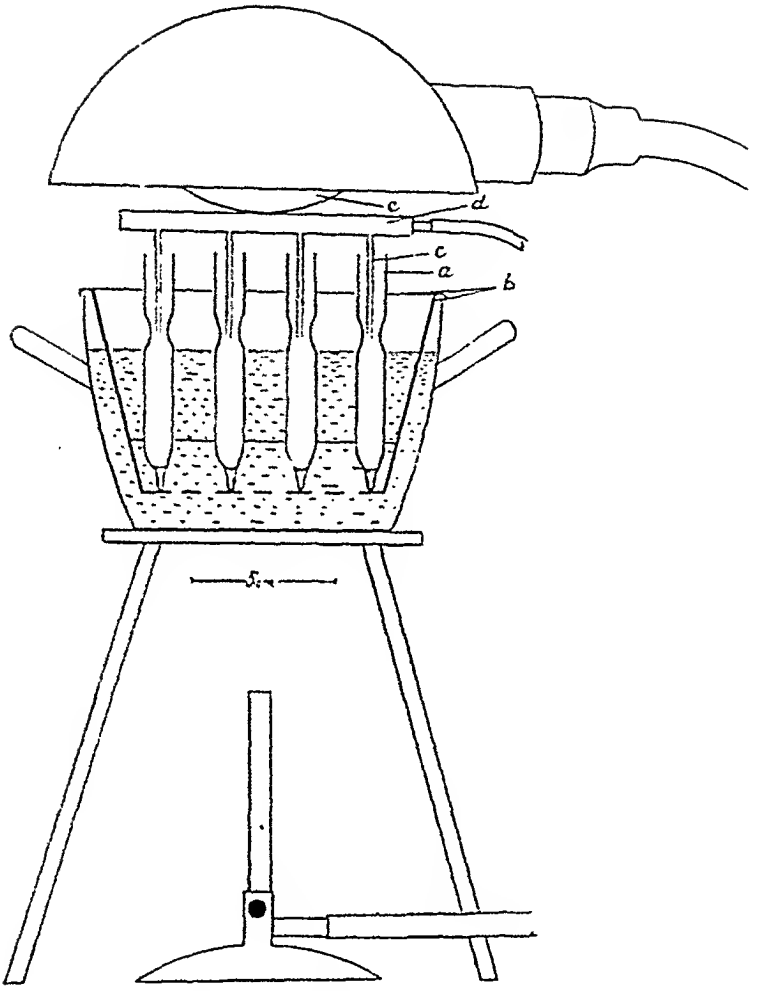


Fig. 1. Apparatus for evaporation.

The analysing vessels (*a*) are placed in a holder (*b*) in a boiling water bath. The air is blown into the vessels through fine cannulae (*c*) of stainless steel (length 35 mm, diameter 0.5 mm), which are fastened to the hollow plate (*d*). The air pressure just before the plate is about 25 mm Hg. In order to prevent condensation of water on the cannulae, the plate is heated by means of an electric lamp (*e*), resting upon the plate.

conical part of the tube ends as a small pocket. In this pocket the precipitate will pack down during the centrifugation, and the aspiration of the supernatant fluid will be possible without disturbance of the precipitate.

A number of ordinary centrifuge tubes are also used in the method.

An apparatus for evaporation. The apparatus is shown in fig. 1,

as adapted for 12 centrifuge tubes. By blowing air into the centrifuge tubes, while they are heated over a boiling waterbath, evaporation can be finished in a few minutes.

An electric oven. The oven shall be large enough to hold 12 of the special centrifuge tubes, and shall be adjustable to about 430° C.

A Rehberg microburette (REHBERG 1925). The total graduated part is 0.100 ml and is graduated in μl. Tenths of the graduation are estimated.

c. Removal of Proteins.

It is necessary to remove the proteins before calcium determination both in saliva and in serum (SCHMIDT-NIELSEN 1944), if this is not done, too low results are obtained. Further it is objectionable to centrifuge the saliva before the samples are pipetted off (l. c.). Some authors have treated the saliva in this way in order to remove the insoluble parts (epithelial cells etc.), but this so called "debris" contains also calcium compounds which are precipitated in the saliva after the secretion. On the contrary it is very important that the saliva is intensively mixed before the pipetting.

Procedure: 1. *Serum.* 0.3 ml of serum are pipetted (Carlsberg pipette) into an ordinary centrifuge tube. 1 ml of distilled water and 0.3 ml of trichloracetic acid are added. The material is mixed and allowed to stand for half an hour for precipitation. The mixture is centrifuged for 10 minutes. With a Carlsberg pipette 1 ml of the supernatant solution is transferred to the special centrifuge tube (the analysing vessel). 2. *Saliva.* The saliva sample is thoroughly stirred, 1 ml is pipetted (Carlsberg pipette) into an ordinary centrifuge tube and 0.3 ml of trichloracetic acid are added. Dilution with distilled water is not necessary here because saliva only contains small amounts of proteins. The material is mixed and treated as described above.

d. Precipitation.

The precipitation of calcium oxalate shall be carried out at a pH between 4 and 5.6 (MCCRUDDEN 1911). If the solution is more acid than pH 4, the precipitation of calcium oxalate will not be complete, and if the pH exceeds 5.6 some magnesium compounds will probably be precipitated together with the calcium oxalate. By neutralizing the trichloracetic acid with ammonium hydroxide and adding an acetate buffer of pH 4.5 the desired pH can be obtained.

Procedure: To the acid solution in the analysing vessel are added 5 drops of brom cresol green solution and 0.2 ml of 1 n ammonium hydroxide. The solution is stirred with a very thin glass rod. If the mixture is still yellow, ammonium hydroxide is added drop by drop until the colour changes from yellow to green or faint blue. If the mixture is blue after adding 0.2 ml of ammonium hydroxide, 1 n hydrochloric acid is added dropwise until the colour changes to yellow, and then one drop of ammonium hydroxide.

After the neutralization 0.5 ml of acetate buffer and 0.5 ml of saturated ammonium oxalate are added, the latter with a syringepipette. If the piston in the pipette is pressed down with a little vigour, no other stirring is necessary.

The tube is stoppered and the mixture is allowed to stand three hours or overnight for precipitation.

e. Washing of the Precipitate.

Removal of the supernatant fluid can be carried out in different ways as decantation, filtration or suction. Here the washing by suction as indicated by HALVERSON and BERGEIM (1917) has been adopted with some modifications. The pipette used for suction can be made as follows: a glass tube is drawn out to a capillary which is bent back into a U (see fig. 3). The suction is car-

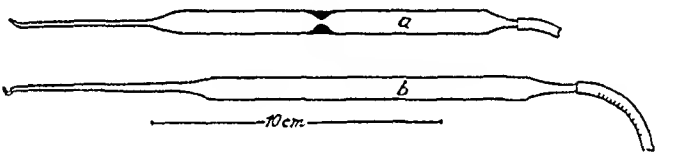


Fig. 2. Pipettes.
A Carlsberg pipette (*a*). The suction pipette (*b*).

ried out with the mouth through a rubber tube. The precipitated calcium oxalate is a light powder which is very easily disturbed during the removal of the supernatant solution. Not even the special form of the centrifuge tube with the small pocket will quite protect the precipitate from being whirled up by the suction, if this is not carried out with the utmost care. Furthermore a film of calcium oxalate will remain in the surface after the centrifugation. Therefore the tip of the suction pipette must always be kept below the surface.

The washing liquid is a 0.5 per cent ammonium oxalate solution. This can be used because the excess of oxalate is volatilized during ignition. The oxalate ions will protect the precipitate from going into solution.

Only one washing is necessary because, after the first suction, not more than 20 μl will be left of the 2 ml mother liquor, these 20 μl will be diluted with 2 ml of 0.5 per cent ammonium oxalate, and again only 20 μl of this mixture will be left by the next suction, so that at most 0.01 per cent of the mother liquor will remain with the precipitate.

Procedure: The mixture is centrifuged for 5 to 10 minutes (3,000 rev/min). With the pipette described above the supernatant fluid is drawn off. The tip of the pipette is immersed into the solution, care must be taken not to touch the walls or the surface during the suction. The solution is drawn off until fluid remains only in the pocket of the tube. 2 ml of 0.5 per cent ammonium oxalate is added. The tube is stoppered and the mixture is again centrifuged for 5 to 10 minutes. After the second suction the remaining fluid is evaporated to dryness in the apparatus for evaporation.

f. Conversion of the Oxalate into Carbonate.

The temperature at which the change from oxalate to carbonate takes place is about 300° C, at a higher temperature some of the carbonate will change to oxide. For the titration results it will not matter if the oxalate is converted into carbonate or oxide or a mixture of both, as the acidimetric equivalent of calcium oxide is identical with that of calcium carbonate. But if not all the oxalate is converted, too low results will be obtained.

At a temperature about 430° C the conversion will be complete in 40—60 minutes. If the temperature exceeds 500° C, or if the analyses are kept more than $1^1/_2$ hour at the high temperature, the hard glass tubes will begin to decompose.

Procedure: The analysing vessels are placed in the electric oven, which is already heated to 430°. After this temperature is reached again, they are allowed to stay in the oven for 40—60 minutes.

g. Solution and Titration of the Calcium Carbonate.

Sobel and Sklersky (1938) have found that hot 10 per cent boric acid will dissolve the calcium carbonate in a few minutes. On account of the special form of the analysing vessel with the small pocket, stirring with a glass rod is necessary. If the glass rod is thin enough and the samples are arranged so that the weakest samples come first and the stronger last, the error produced by transporting fluid from one sample to another will be negligible.

The indicator used for the titration is brom cresol green. The pH range is 3.8—5.4, the colour changes from yellow to blue.

When the solution of calcium carbonate in boric acid is titrated to the pH of the pure boric acid, the hydrochloric acid used for the titration exactly equivalents the calcium content of the sample.

Procedure: After the ignition the analysing vessels are placed in the evaporation apparatus, which is now used only as a boiling water bath. 0.5 ml of hot 10 per cent boric acid is added. The pipette must be washed immediately after the use to avoid crystallization inside it. With a very thin glass rod each sample is stirred for 10 sec. The samples remain for a few minutes in the boiling water bath, then they are taken out and 2 ml of indicator solution are added. The samples are allowed to cool to room temperature, and the titration can take place.

For comparison 0.5 ml of the boric acid are pipetted into a cleananalysing vessel and 2 ml of indicator solution added. The REHBERG microburette is filled with 1/10 n hydrochloric acid just before use. The tip of the burette is immersed in the solution. For stirring a gentle stream of air is bubbled through. The titration is finished when the sample has obtained the same colour as that of the pure boric acid + indicator.

h. Calculations.

The calcium content in the sample can be expressed in a) millinormality (= μE of Ca per ml of sample) or in b) mg of Ca per 100 ml of sample.

When we wish to calculate the calcium content in millinormality we have:

1 μl of $^1/_{10}$ n HCl corresponds to $^1/_{10}$ μE Ca

then

$$\mu\text{E of Ca per ml of sample} = \frac{(\text{titer} - \text{blank}) \cdot {}^1/_{10}}{\text{ml of sample}} = \text{millinormality of sample.}$$

If the calcium content is to be calculated in mg per 100 ml of sample we have:

1 μl of $^1/_{10}$ n HCl corresponds to 0.002 mg Ca

then

$$\text{mg of Ca per 100 ml of sample} = \frac{(\text{titer} - \text{blank}) \cdot 0.002 \cdot 100}{\text{ml of sample}}.$$

When serum has been treated with trichloracetic acid, we have that the quantity of serum first pipetted off (ser) has been diluted with water (wa) and with trichloracetic acid (tri), and, after precipitation and centrifugation, an aliquot part of this mixture (pip) has been transferred to the analysing vessel. The quantity of serum transferred to the analysing vessel (ml of sample) can then be calculated as follows:

$$\text{ml of sample} = \text{pip} \cdot \frac{\text{ser}}{\text{ser} + \text{wa} + \text{tri}}.$$

When a sample of saliva has been treated with trichloracetic acid, the procedure has been the same as that for serum except the diluting with water. If "sal" is the quantity of saliva first pipetted off, the quantity transferred to the analysing vessel can be calculated as follows:

$$\text{ml of sample} = \text{pip} \cdot \frac{\text{sal}}{\text{sal} + \text{tri}}.$$

Experimental.

The method has been used in daily routine work for one and a half year. All this time the results of control analyses and parallels have been satisfactory. Some characteristic analytical data shall be given below.

It is necessary to make blank analyses. If the reagents are not quite free from traces of calcium, the blanks will give a small titer, which must be subtracted from the titer of the total sample.

Table 1.

Blanks.

The results are expressed in μl of HCl used for the titration.

H_2O	H_2O + trichloracetic acid
0.1	0.5
0.0	0.5
0.0	0.5
0.0	0.5
0.1	0.6
0.0	0.5

In table 1 the results on distilled water and on water treated with trichloracetic acid are shown.

Table 2.

$CaCl_2$ solution of known strength. 4 mg of Ca in 100 ml of solution.

$CaCl_2$ treated with trichloracetic acid 0.790 ml analysed = 0.0316 mg Ca.	$CaCl_2$ not treated with trichloracetic acid 1.080 ml analysed = 0.0432 mg Ca.
0.0314	0.0430
0.0313	0.0433
0.0316	0.0428
0.0317	0.0433
0.0314	0.0432
0.0317	0.0429
0.0313	0.0435
0.0316	0.0430
0.0317	0.0431
0.0320	0.0434
Standard deviation 0.000224	Standard deviation 0.000234
Percentage error 0.71	Percentage error 0.54

In table 2 the results on determinations of calcium chloride solutions of known strength are shown. Preliminary treatment with trichloracetic acid does not seem to influence the standard deviation. The percentage error is about 0.6.

Table 3.

Saliva.

Eight analyses of one sample of saliva.

mg of calcium found in 0.810 ml saliva
0.0491
0.0493
0.0487
0.0485
0.0489
0.0485
0.0490
0.0489
Average: 0.0489
mg of calcium in 100 ml of saliva: 6.04
Standard deviation 0.00033
Percentage error 0.68

Table 4.

Serum.

Eight analyses of one sample of serum.

mg of calcium found in 0.244 ml serum
0.0256
0.0259
0.0252
0.0249
0.0249
0.0254
0.0258
0.0258
Average: 0.0254
mg of calcium in 100 ml of serum: 10.4
Standard deviation 0.00040
Percentage error 1.6

Tables 3 and 4 give the results from a saliva and a serum sample. The standard deviation is a little larger for these protein containing solutions than for the pure calcium chloride solution. The percentage error is larger for the serum than for the saliva samples, owing to the smaller quantity of calcium analysed.

In order to control that the presence of saliva does not influence the correct precipitation of the calcium present, mixtures of saliva and a calcium chloride solution of known strength have been analysed. The results are seen in table 5. The calcium contents found by analysis and by calculation are the same.

Table 5.

Mixtures of saliva and $CaCl_2$ solution.

	mg of Ca in 100 ml of sample	
	analysed (average of 4 analyses)	calculated
Saliva	5.56	
2 parts saliva + 1 part $CaCl_2$	5.08	5.09
1 part saliva + 1 part $CaCl_2$	4.85	4.83
1 part saliva + 2 parts $CaCl_2$	4.67	4.60
$CaCl_2$	4.10	

Summary.

A method for determination of calcium in about 0.3 ml serum or 1.0 ml saliva (0.02 to 0.05 mg of calcium) is reported. The principle is the same as that indicated by SOBEL and SKLERSKY, but the procedure is somewhat different. The duration of the procedure has been shortened and the accuracy has been increased by the introduction of a number of improvements.

The principle is: The calcium is precipitated as oxalate. The oxalate is converted by ignition to carbonate, dissolved in a hot solution of 10 per cent boric acid, and titrated with a 1/10 n hydrochloric acid to the pH of the pure boric acid.

When 24 samples are treated together the procedure requires 3 hours up to and including the precipitation and 3 hours more for the final treatment and the titrations. The percentage error of the single determination is about 0.7 for 0.05 mg of calcium.

References.

FISKE, C. H., and E. T. ADAMS, J. Amer. Chem. Soc. 1931. *53*. 2498.
HALVERSON, J. O., and O. BERGEIM, J. biol. Chem. 1917. *32*. 159.
HAMILTON, B., Ibidem. 1925. *65*. 101.
HOLTER, H., C. R. Lab. Carlsberg Sér. chim. 1943, *24*. 399.
KATZMAN, E., and M. JACOBI, J. biol. Chem. 1937. *118*. 539.
KRAMER, B., and F. F. TISDALL, Ibidem. 1921. *47*. 475.
KROGH, A., Ind. Eng. Chem. 1935. *7*. 130.
RAPPAPORT, F., Klin. Wschr. 1933. *12*. 1774.
REHBERG, P. B., Biochem. J. 1925, *19*. 270.
SOBEL, A. E., and S. SKLERSKY, J. biol. Chem. 1938. *122*. 665.
SOBEL, A. E., and B. A. SOBEL, Ibidem. 1939. *129*. 721.
TREVAN, J. W., and H. W. BAINBRIDGE, Biochem. J. 1926. *20*. 423.
WILLARD, H. H., and P. YOUNG, J. Amer. Chem. Soc. 1920. *50*. 1322.

From the University Institute of Medical Physiology, Copenhagen.

The Phosphorylation of Glucose in Liver Extracts.

By

JINGE KJERULF-JENSEN.

Received 25 October 1944.

LUNDSGAARD, NIELSEN and ØRSKOV in 1936 found that an isolated cat liver when perfused with blood containing glucose is incapable of assimilating the glucose, whereas an isolated rabbit liver under the same conditions does so. Fructose, when administered under similar experimental conditions, was assimilated readily by both cat and rabbit livers.

Subsequently KALCKAR, 1938, in a single experiment observed that during incubation with glucose the fluoride-poisoned extract of liver tissue from a starved cat consumed inorganic phosphate, and this he regarded as a manifestation that the added glucose was phosphorylized by the enzymes of the liver tissue.

However, if we assume that all hexose assimilation in the liver is conditioned by a phosphorylation process, it would seem that there is some incongruity between the observed glucose phosphorylation in vitro and the failure to assimilate glucose in vivo in the cat liver. The present work attempts to throw some light on this incongruity, the author having examined to what degree glucose and fructose can cause phosphorylation in fluoride-poisoned extracts of livers of cats and rabbits, as well as of rats.

Technique.

Preparation of liver extract. The animal was kept in a state of inanition for 24 hours prior to the experiment. The liver was removed under ether narcosis, cooled on ice for 20 minutes, weighed, and then pulped

with sand and an equal weight-volume of extraction fluid, i. e. $^1/_{15}$ molar potassium-sodium phosphate solution with pH 7.3 containing 2 per cent. sodium fluoride and 0.3 per cent. potassium chloride. Phosphate was necessary, on account of the absorption of phosphate during the phosphorylation process and as a buffer substance. The purpose of the sodium fluoride was to inhibit dephosphorylation and thus make possible an accumulation of the phosphorylized products. Potassium chloride was added for the purpose of imitating the intracellular concentration of these substances.

Incubation proceeded under a supply of oxygen for 30 minutes in a water bath at 37°.

After incubation the proteins were removed with 10 per cent. trichloroacetic acid. The concentration of inorganic phosphate in the filtrate was determined colorimetrically after a modification of LOHMANN and JENDRASSIK's method.

To each sample of tissue extract was added $^1/_{15}$ volume of either 2 per cent. glucose or fructose or water, respectively. The loss of phosphate owing to incubation without a substrate represents the phosphorylation of the preexisting substances in the liver extract; this value is called the spontaneous phosphorylation. The loss of inorganic phosphate beyond this spontaneous phosphorylation represents the phosphorylation of glucose and fructose respectively.

Table 1.

Phosphorylation i. e. phosphate decrease in fluoride-poisoned liver extracts from cat, rabbit and rat during incubation with solutions of glucose or fructose or with water alone. Experimental conditions: see text. The figures represent inorganic phosphate as mg P per sample.

Animal No.	Phosphate loss when incubated without substrate	Extra phosphate loss when incubated with glucose	Extra phosphate loss when incubated with fructose
Cat 1	0.31	0.05	1.03
2	0.65	0.06	0.39
3	1.05	0.00	0.19
4	1.25	0.03	0.21
5	1.46	0.08	0.47
6	1.48	0.04	0.40
Rabbit 1	0.17	0.11	0.17
2	0.62	1.44	0.76
3	0.76	0.11	0.17
4	1.70	0.20	0.12
Rat 1	0.05	0.03	0.03
2	0.27	0.09	0.16
3	0.50	0.10	0.10
4	0.86	0.20	0.35

Results of Experiments.

An examination of the table shows that on being incubated without a substrate the phosphorylation in the liver extracts from the three different animals is considerable. This must indicate that the liver tissue contains abundant quantities of preexisting substances which can be phosphorylized. The author is aware that this considerable spontaneous phosphorylation makes it difficult to interpret the results of the experiments. Attempts to reduce the spontaneous phosphorylation for example by starving the animal were unsuccessful. Under the various experimental conditions this spontaneous phosphorylation is always of considerable magnitude and varies irregularly from animal to animal; thus the author did not succeed in removing the preexisting substances by a dialysis of the medium prior to the phosphorylation experiment.

When incubated with glucose there was a very slight increase of phosphorylation in the liver extracts from cats, whereas those from rabbits and rats assimilated perceptibly more phosphate when incubated with glucose. On the other hand the cat liver extracts phosphorylized distinctly when incubated with fructose. For rats and rabbits there was no distinct quantitative difference in the uptake of phosphate when incubated with glucose and fructose respectively. According to KALCKAR 1938 the phosphate ester formed during the incubation of liver extract with fructose is a fructose-diphosphoric acid; it would thus seem that there is an esterification of the hexose itself. If we may venture to draw conclusions from conditions in vitro compared with conditions in vivo, the inability of the cat liver tissue to assimilate glucose must mean that in this animal the liver tissue does not contain a sufficient quantity of enzymes for the phosphorylation of glucose. The possibility that glucose actually inhibits spontaneous phosphorylation in the liver tissue seems out of the question, as after incubation the concentration of glucose was found to have no relation to the extent of the spontaneous phosphorylation.

Even with an intense stimulation of the phosphorylation in the cat liver extracts through the addition of glutamic acid there was no observable increase in the glucose phosphorylation, as a sample of the extract with glutamic acid and water phosphorylized

just as much as a corresponding extract with glutamic acid and glucose. Thus the increased phosphorylation caused by adding glutamic acid is due solely to an increased phosphorylation of the preexisting substrates, i. e. spontaneous phosphorylation. When stimulating phosphorylation in kidney cortex extracts of rabbits and cats KALCKAR 1938 and KJERULF-JENSEN 1942 respectively found that there was esterification not only of the preexisting substances but also of added glucose or glycerine. It is a well-known fact that the oxydation of several different easily-oxidizable organic compounds, especially polycarbonic acids like citric acid, succinic acid, including glutamic acid, can energetically stimulate the phosphorylation of hexose in phosphorylizable, respiring tissue extracts. Thus the conditions for a phosphorylation of glucose in a tissue extract of cat liver may be described as the best possible when, simultaneously with incubation, the added glutamic acid is broken down.

Working on the idea that cat liver tissue lacked some component of the enzyme system necessary to the phosphorylation of glucose — a component that is not required for the phosphorylation of fructose — the author endeavoured to complete the liver extracts by adding small quantities of aqueous kidney cortex extract from the same animal. In a fluoride-poisoned state these extracts are capable of phosphorylizing glucose very rapidly, so that they must contain the components necessary for this process. Before being added to the liver extracts the phosphorylizing enzymes in the renal cortex extracts were destroyed very gently by placing them in the thermostat at 35° for 5 to 10 minutes without any previous addition of fluoride. However, this attempt to complete the extract of cat liver tissue failed, as there was no significant phosphorylation of glucose.

It would thus seem that the inability of cat liver to phosphorylize glucose is due to the absence of certain very labile components, presumably the phosphorylizing enzymes which are specifically directed against glucose.

Summary.

By means of phosphorylation experiments with liver extracts from cats, rabbits and rats it was found that as far as cat liver extract is concerned, incubation with glucose — in contrast to the other animals — caused only an insignificant assimilation of

phosphate. This agrees with LUNDSGAARD, NIELSEN and ØRSKOV's demonstration that artificially perfused cat liver does not assimilate glucose from the perfusion blood.

The experiments also indicated that the inability of cat liver tissue to phosphorylize glucose is caused by a lack of the phosphorylizing enzymes that act specifically on glucose.

References.

KALCKAR, H., Fosforyleringsprocesser i dyrisk Væv, Diss., København 1938.

KJERULF-JENSEN, K., Fosforyleringsprocessers Betydning for Resorption. Diss., København 1942.

LUNDSGAARD, E., N. AA. NIELSEN and S. ØRSKOV, Skand. Arch. Physiol. 1938. *73*. 296.

From the Biological Institute of the Carlsberg Foundation.

Protein Metabolism of Tissue Cells in vitro

3. Accessory Growth Substances Present in Barley Malt

By

TAGE ASTRUP and ALBERT FISCHER

Received 7 November 1944.

In a previous paper (ASTRUP, FISCHER and VOLKERT 1945) it was demonstrated that thermolabile dialyzable accessory growth substances for animal tissue cells are present abundantly in extracts of yeast and germinating barley. These substances appeared to be of the same nature as the low molecular substances present in blood serum required for maintenance and growth of the cells in the plasma medium. The results obtained in these studies indicated that only a single or a few substances were responsible for the effect, which possibly may be to enable the cells to utilize the dialyzed plasma proteins.

In the present paper the consequences of these results are drawn, attempts being made, with germinating barley as raw material, to isolate fractions that may be able without the presence of other substances (*e. g.*, amino acids as employed previously) to elicit normal growth of tissue cultures in dialyzed media. Our experiments show that actually it is possible to produce purified preparations which still have the full effect of the original extract, thus supporting the assumption that a single or only few active components are involved.

Technique.

The technique described in previous papers was employed here, except that this work was carried out without any addition of other substances. Thus no amino acids were added. Furthermore, the ex-

periments were carried out almost exclusively with the employment of heart fibroblasts from chicken embryos, while before usually periosteal fibroblasts (osteoblasts) were used. Periosteal fibroblasts respond very readily to the addition of the accessory growth substances and require the presence merely of relatively small amounts of growth substance in the form of dialyzed embryonic extract (embryonin). Using osteoblasts, the result of the experiment can be seen as early as after 3—4 days.

In heart cultures a larger amount of the accessory substances originating in the mother culture is transferred to the experimental cultures, and during the first 24—28 hours the culture therefore grows better than is to be expected on a dialyzed medium. With heart fibroblasts it therefore takes 5—8 days before a reliable result may be obtained from the experiment. Various experimental series, in which the effect of the same extract was examined on osteoblasts and heart fibroblasts, showed that one obtains just as reliable experimental results with heart fibroblasts as with osteoblasts.

It may be added that these studies so far did not reveal any differences in the demand for accessory growth substances between osteoblasts and heart fibroblasts, except that the heart fibroblasts, for the full rate of growth, require larger amounts of these substances than do the osteoblasts.

The following experiments have as a rule been carried out with heart fibroblasts and with addition of larger amounts of accessory substances than employed in the previous experiments, namely: 0.2 and 0.4 ml. It was found convenient, in order to obtain reliable results to carry out at the same time experiments with 0.2 and 0.4 ml of the solution concerned. As is well known, the response of the cultures to the substances added (embryonic extract and dialyzable factors) may vary from one culture to another. Hence it cannot be taken for granted that one set of cultures (an experimental culture with the corresponding control culture as daughter cultures from the same mother culture) respond to the addition of embryonic extract and accessory substances to the same degree as does another set. Hence, with the measuring method here employed, no absolute values can be given for the experimental results.

The solutions examined were usually prepared with saline or Ringer's solution and adjusted to neutral reaction on neutral lithmus paper, whereafter they were sterilized by passing through asbestos filters.

Experimental.

The raw material used was barley malt (pilsner malt) dried at 90°, placed at our disposal by the *Carlsberg Breweries*. It was ground to a powder and the main amount of husk was removed by sifting.

1. *Crude Extract.* — 200 g pulverized malt is added to 500 ml water at 35—40° and stirred on water bath at this temperature

for one hour. The mixture is centrifuged, and the turbid supernatant fluid (250—300 ml) is precipitated with 3 vol. 93—96 per cent ethyl alcohol and left standing for a couple of days. The filtrate herefrom is evaporated in vacuum on a water bath till all the alcohol is removed, and is then diluted with water to the original volume (250—300 ml). Contains 0.80—1.00 mg N per ml.

2. *Alkaline Precipitation with Ethyl Alcohol.* — As previous experiments with yeast extracts show that fractional precipitation may be carried out with ethyl alcohol, the behavior of malt extracts was examined — with corresponding results: Alcohol precipitates a *neutral or alkaline* concentrate so that the amount of nitrogen is distributed about equally on the two fractions, while the *active substances* are found *in the precipitate.* On the other hand, with *acid reaction* of the concentrate the amount of nitrogen in the precipitate is decreasing and, at the same time, the *solution becomes* more and more *active.* Several of the experiments were carried out on crude extract treated with fullers earth, but as only small amount of the nitrogen is removed, this treatment was later omitted. Precipitation at basic reaction gives the most promising result, since all the active substances are found in the precipitate. Still, the solution produces some growth, but the appearance of the cells shows that we are here dealing with a very inadequate medium.

V—212, 1: 300 ml crude extract, prepared as above, is concentrated in vacuo on a water bath to 23 ml and rendered slightly basic on phenolphthalein paper by addition of 2-n NaOH. The amount is now 28 ml. This is precipitated with 5 vol. 96 per cent ethyl alcohol (140 ml) and left standing till next day at 0°.

The supernatant fluid is distilled in vacuo on a water bath with water in order to remove the alcohol. Neutralization and dilution to the original volume with water. 0.41 mg N/ml.

V—212, 2: The precipitate is dissolved in water and the solution distilled in vacuo in order to remove the alcohol. Dilution to the original volume, after neutralization. 0.50 mg N/ml.

V—212, 2 is considerably more active than V—212, 1; in particular, the appearance of the cells shows that V—212, 1 is insufficient, Figs. 1 and 2. About one-half of the amount of nitrogen is removed in inactive form. Repetition of the precipitation gives a further purification.

V—217, 1: 560 ml crude extract is concentrated to a small volume and made basic as above. Volume 68 ml. Precipitation with 5 vol. 96 per cent alcohol and standing at 0° till next day. Decantation of supernatant fluid and dilution to 560 ml. Contains 0.39 mg N/ml.

V—217, 2: The precipitate is dissolved by addition of 25 ml water

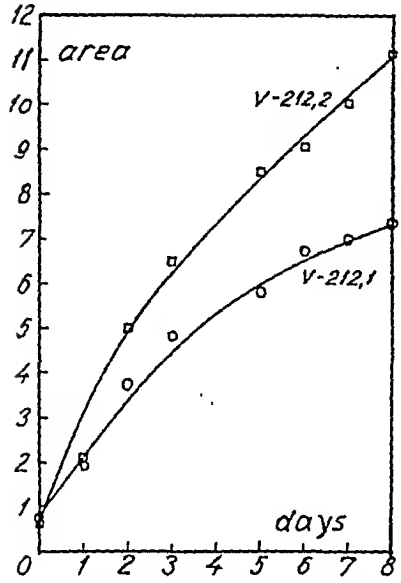


Fig. 1. Growth of tissue cultures on dialyzed media containing accessory growth substances from barley malt (V—212, 1 and V—212, 2) isolated by precipitation with alcohol at basic reaction.

and reprecipitated with 250 ml 96 per cent alcohol. After standing til next day the fluid is poured off and diluted to 560 ml. 0.17 mg N/ml.

V—217, 3: The precipitate from V—217, 2 is dissolved in water, distilled in vacuo, neutralized and diluted to 560 ml. Contains 0.32 mg N/ml.

The solution purified by two precipitations with alcohol at alkaline reaction appeared to be just as active as the crude extract and was used for further purification.

3. *Acid Precipitation with Ethyl Alcohol.* — A concentrate (of a crude extract or a solution purified by basic precipitation with alcohol) is found to dissolve in excess of glacial acetic acid. By fractional precipitation with ethyl alcohol, it is possible to obtain an inactive nitrogen-poor precipitate. On further addition of ethyl alcohol an increasing amount of nitrogen and active substance is found in the precipi-

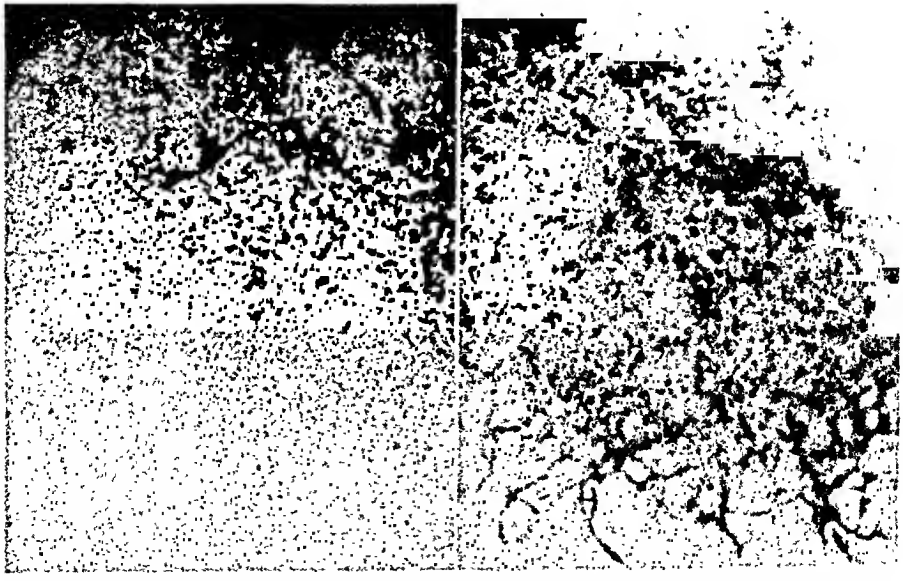


Fig. 2. The same cultures as in Fig. 1, photographed on the 5' day of growth. Magnif. 40 ×. (V—212, 2; V—212, 1.)

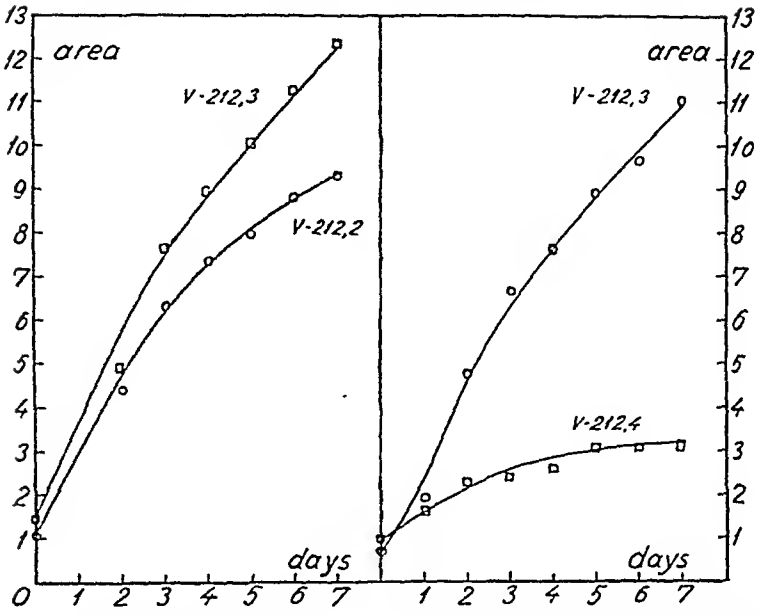


Fig. 3. Treatment of the crude extract with glacial acetic acid and ethyl alcohol (V—212, 2: Original solution. V—212, 3: Filtrate. V—212, 4: Precipitate).

tate, but even with a large excess of ethyl alcohol it is not possible under these circumstances to precipitate all the active substances in the solution. The most favorable result is obtained by precipitation with just as much absolute alcohol as the amount of glacial acetic acid used. The precipitate obtained in this manner is very low in nitrogen and practically inactive, whereas the solution appears to be just as active as the original solution. Further alcoholic fractionation of this solution gives no satisfactory result.

V—212, 3: 200 ml of V—212, 2 is concentrated in vacuo to a thin syrup (17 ml) and 5 vol. glacial acetic acid (85 ml) is added; then precipitation with 5 vol. absolute alcohol (85 ml), and standing overnight at 0°. The precipitate is a little sticky. The clear fluid is poured off, and distilled in vacuo with water several times in order to remove the acetic acid. Neutralization and dilution to 200 ml 0.39 mg N/ml.

V—212, 4: The precipitate is dissolved in water and distilled in vacuo for removal of the acetic acid. Neutralization and dilution to 200 ml 0.07 mg N/ml.

While the supernatant fluid appears to be just as active as the original solution, the precipitate is almost inactive (cf. Fig. 3). By this operation only a small amount of nitrogen is removed, but, by weight, a considerable amount of substance has been eliminated, presumably consisting of carbohydrates.

4. *Extraction with Phenol.* — When the aqueous solution of the active substances is shaken with phenol, all the activity is found in the aqueous layer, while the phenol layer is inactive. At the same time, the phenol has removed practically all coloured substances from the solution, which now is only faintly yellowish. Also an impurity is removed which makes the solution foam markedly at the evaporation in vacuum.

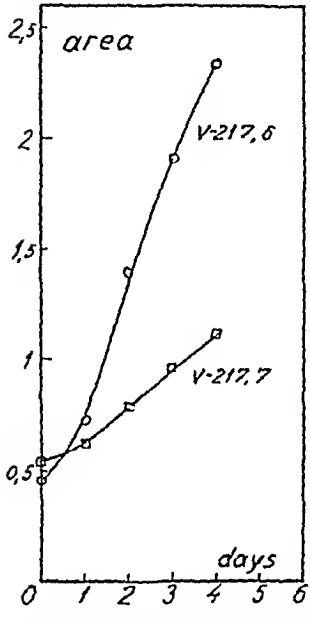


Fig. 4. Treatment of the extract with phenol. V—217, 6: Aqueous layer, V—217, 7: Phenol layer).

V—217, 6: 80 ml concentrate, corresponding to 400 ml crude extract purified through two basic alcohol precipitations and one treatment with glacial acetic acid and ethyl alcohol, is shaken twice with 40 ml 90 per cent phenol. The water layer, which now is light yellow, is shaken with 100 ml ether. Then distillation in vacuo with several additions of water for removal of the ether and phenol. Neutralization and dilution to 400 ml. Contains 0.16 mg N/ml.

V—217, 7: The united reddish-brown phenol layers are mixed with 100 ml water, and the mixture is shaken with 150 ml ether. As the separation is slow, the mixture is left standing till next day. The aqueous layer is removed, and the phenol-ether is shaken twice with more water and ether. The aqueous layers are distilled in vacuo, foaming strongly under this treatment. Evaporation to dryness several times for removal of traces of phenol. Neutralization and dilution to 400 ml. Contains 0.23 mg N/ml.

Here the purification process has been quite effective, as hardly one-half of the total amount of the nitrogen is left in the highly active aqueous phase. The phenol layer is inactive. (See Fig. 4.)

Discussion.

By the treatments described above, a considerable purification of the accessory growth substances is obtained, and as the activity still follows but *one* fraction it becomes increasingly probable that a single or only a very few substances are responsible for the effect on the tissue cells. While the crude extracts contain from 0.80 to 1.00 mg N per ml, the most purified solution (diluted to a corresponding volume) contains but 0.16 mg N

per ml or even less, apparently without any essential reduction in the effect. At any rate, the fall in activity is not greater than is to be expected with the unavoidable loss of active substance in the treatment of the extract. Measured by its nitrogen content, the active substance has been concentrated 5—8 times. The purification is considerably more effective, however, as fairly large amounts of substances low in nitrogen are removed. This is evident from dry matter determinations on the solutions prepared (105° to constant weight).

With the various forms of treatment, 500 g crude extract gives approximately the following amounts of dry matter:

Crude extract, originally	30 g
After two precipitations with basic alcohol	12 g
After precipitation with acetic acid and ethyl alcohol	6 g
After treatment with phenol	2.5 g

Calculated on dry matter content (before dilution or addition of Ringer's solution or saline, but including the neutralizations required) the last preparation is about 12 times more active than the original crude extract.

The experiences from these processes of purification have confirmed the strong hydrophilic properties of the active substances. Other properties are the following:

They are dialyzable and thermolabile in aqueous solution. In dilute aqueous solution they are not precipitated by 3 vol. 96 per cent alcohol. After concentration they are precipitated completely at neutral and basic reaction by 96 per cent alcohol. At acid reaction their precipitation decreases with increasing acidity of the solution. The active fraction is soluble in 90 per cent acetic acid. In such solution, one volume of absolute alcohol produces a crystallized inactive precipitate which contains only minimal amounts of nitrogen. The filtrate contains all the active substances. Addition of more absolute alcohol gives increasing amounts of nitrogen in the precipitate which at the same time becomes more and more active, but even a large excess of absolute alcohol does not give complete precipitation of the active substance. On shaking with phenol the active substance remains in the aqueous phase while almost all coloured substances and the foaming substance are transferred to the phenol phase.

Experiments were carried out in order to isolate an active product in dry form by concentration in vacuo of the solution

purified by phenol extraction, followed by neutralization and precipitation with alcohol. In this way a sticky precipitate was obtained which could be transferred to a dry state by treatment in a mortar with alcohol and dry ether. In the air, however, this dry substance again becomes syrupy, and assumes a brownish colour. It is strongly hygroscopic, and is best kept under alcohol.

Summary.

Extracts of barley malt, containing accessory growth substances for animal tissue cells, are submitted to fractionation, which results in a considerable purification and gives some information about the properties of the active substances.

The studies here reported were carried out with the aid of grants from *King Christian X's Fond* and *Danmarks tekniske Højskoles Fond for teknisk Kemi.*

Reference.

ASTRUP, T., A. FISCHER and M. VOLKERT, Acta Physiol. Scand. 1945 (in press).

From Arbetsfysiologiska Undersökningen, Stockholm.

A Photoelectric Pulse Counter.

By

NILS P. V. LUNDGREN and YNGVE ZOTTERMAN.

Received 22 November 1944.

In physiological investigations concerning bodily heavy work there has always been a strong demand for a suitable method of recording the heart rate during exercise. For that purpose the electrocardiogram has been recorded of which sometimes all other waves but the R-wave have been filtered off. Lately LEHMANN (1944) mentions that MÜLLER has made a photoelectric pulse counter, which is arranged on the ear lobe and which has been proved to be valuable in research on industrial work. He does not give any details about the amplifier or recording means. It gave us, however, the idea to make a simple pulse counter which could be used in our investigations upon lumbermen in the woods.

The photocell used consists of a selenium barrier layer cell of Pressler's manufacture. The smallest design has a diameter of 17 mm and a surface of 3 cm^2, which at an external resistance of 2 000 ohms gives 120×10^{-6} amps. at an illumination of 1 lumen. The cell is fixed on the outside of the earlobe by means of clip arrangement. A small lamp fed from an ordinary dry cell battery is fixed on the other branch of the clip (fig. 1 a). By means of a 6 meter long cable the photocell is connected to the input of a 4-stage amplifier driven entirely by the dry-cell batteries. The coupling of this amplifier is given in fig. 1 b. This amplifier works a polarized relay, which controls either a telegraph receiver recording each pulse beat, or, as in fig. 1 c, two call meters, which can successively be brought into play by a switch.

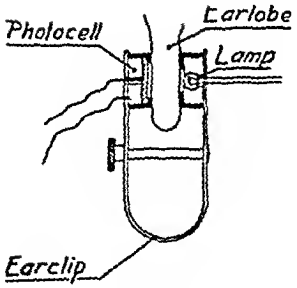


Fig. 1 a.

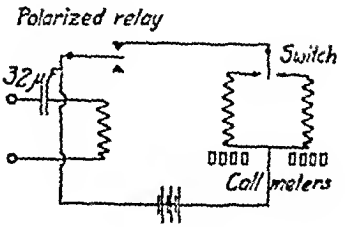


Fig. 1 c.

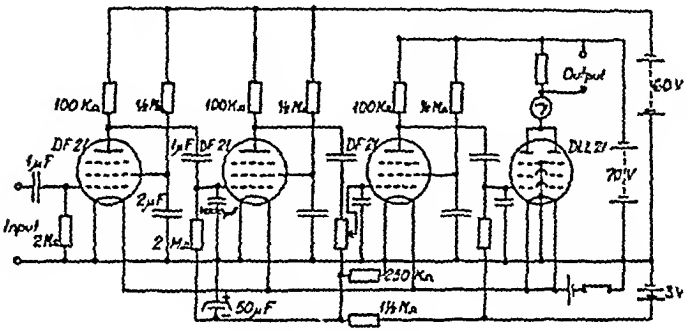


Fig. 1 b.

This arrangement has proved to work satisfactorily during for instance cross cutting of logs and has been used during measurements of the oxygen consumption upon lumbermen in order to work out diagrams relating the heart rate to the rate of oxygen consumption. It works fairly satisfactorily but for very jerky movements of the head. In such cases as for instance while cutting branches with the axe it is advisable to count every deflection of the milliameter which is inserted into the anode circuit of the last valve. By this counting one gets into the rhythm of the pulse and the irregularities caused by occasional strong jerks of the head can thus be corrected for. In experiments on a bicycle ergometer the pulse counter was found to work very well even up to performances of 1500 mkg/min.

Reference.

LEHMANN, G., Stahl und Eisen 1944. *64*. 85.

From the Laboratory of Zoophysiology, University of Copenhagen.

The Reabsorption of Glycine and Other Amino Acids in the Kidneys of Man.

By

HANS H. USSING.

Received 26 October 1944.

Introduction.

Among the normal constituents of human urine, and for that matter the urine of most vertebrates, amino acids are regularly found. ABDERHALDEN and SCHITTENHELM (1906) isolated glycine from urine, and later histidine (KAPPELER-ADLER, 1933) and cystine (MEDES, 1937) were found to occur normally in human urine. A person voiding 1.5 litre urine per day, containing, say, 15 mg % amino-N thus excretes 225 mg amino-N corresponding to about 1.5 g protein.

This is, however, a very small part of the amino-N which has in the same period passed from the blood into the glomerular filtrate. Assuming the normal amino-N concentration of the blood-plasma to be about 5 mg % and a reasonable filtration in the kidneys to be 150 litres per day, about 7.5 g amino-N passes every day into the glomerular filtrate and, as only a minor part of that amino-N is found in the urine, it is obvious that the major part of the amino acids are reabsorbed in the tubules or possibly have diffused back into the blood as the urine is concentrated by reabsorption of water.

KIRK (1936) studied the excretion of amino-N in fasting persons and in persons who had ingested 25 g portions of glycine. He found a considerable increase in the amino-N clearance with increasing amino-N concentration in the plasma. The observed rise

in the clearance he explained by a reduced tubular reabsorption of amino acids. He found further that the amino nitrogen concentration may at times be lower in the urine than in the plasma. It thus seems beyond doubt that amino acids are reabsorbed by the human tubuli, but little is known concerning the mechanism by which amino acids are reabsorbed.

DOTY (1941) determined the reabsorption in dog's kidneys of l-tyrosine, N-acetyl-l-tyrosine, N-methyl-l-tyrosine and l-histidine. He found that whereas tyrosine and histidine were reabsorbed to a high extent (96—99 %), only 64 % of methyl-tyrosine and practically no acetyl-tyrosine was reabsorbed.

It thus seems as if the mechanism involved is preferably adapted to the amino acids proper. In this connection attention may be drawn to the known fact that the d-amino acids not normally present in the organism, when given in the food are excreted in the urine to a considerable extent, whereas the "natural" l-amino acids are as a rule excreted in traces only (compare ABDERHALDEN and TETZNER, 1935). Of course this excretion might be due to a slow oxydation of the "unnatural" isomeres, but as the organism possesses a very powerful d-amino acid oxydase especially in the kidney (KREBS, 1935) the more likely explanation of the excretion of d-amino acids is that they are not so efficiently reabsorbed as are the natural ones.

An important question in the further study of amino acid reabsorption is whether one mechanism is responsible for the reabsorption of all amino acids or perhaps different amino acids are reabsorbed by more or less specific mechanisms.

In principle this problems may be easily solved. As stated above, KIRK (l. c.) found that a moderate increase in plasma amino-N (produced by glycine ingestion) resulted in a large increase in amino acid excretion. This indicates that the amino acid reabsorbing power of the tubules is limited just as is the power to reabsorb glucose. If one reabsorption mechanism is common to all amino acids, an increase in the plasma concentration of one amino acid should lead to an augmented excretion of all amino acids occurring in the plasma. Similarly, if some mechanism is common to a group of amino acids, the amino acids of that group would of course be excreted in excess if one member of that group were augmented in the plasma.

Though simple in principle such experiments have certain practical limitations. In the first place most l-amino acids are so

rapidly handled in the metabolism that the concentration in the blood cannot be increased measurably, in any case if the amino acid is given by mouth (compare ABDERHALDEN and TETZNER, l. c.). Secondly the prevailing war conditions make it very difficult to get the necessary amounts of most amino acids. From the few accessible amino acids glycine seems best suited for such experiments. It is not so rapidly metabolised by man as most l-amino acids and, according to KIRK (l. c.), it is therefore possible to increase the amino-N of the blood from the fasting value of 5—6 mg % to about 10 mg % by giving the subjects 25 g glycine by mouth. (DOTY and EATON (1939) found that lysine is still more slowly oxydized than is glycine, but the administration of this amino acid to dogs by stomach tube resulted in vomiting).

In evaluating such experiments with glycine it must be remembered, however, that even a reabsorption mechanism common to all amino acids might quantitatively prefer other amino acids for glycine, which would mean that an increase in the glycine content of the blood would probably produce a high excretion of that amino acid, but only a small increase in the excretion of other amino acids. This means that whereas a glycine feeding experiment showing an increased excretion of other amino acids must be taken as a proof for a common reabsorption mechanism, a negative find is inconclusive until more is known about the relative amounts of glycine in serum and in urine.

ABDERHALDEN and SCHITTENHELM (l. c.) isolated glycine from normal urine, whereas they were unsuccessful in isolating any other amino acid, and they therefore assumed glycine to be the only amino acid normally occurring in urine.

Although certain other amino acids have since been shown to be present in normal urine (see above), it cannot be ascertained from the literature if glycine is the all dominating amino acid in urine or whether other amino acids are of similar importance.

Similarly, several amino acids are known to occur in blood plasma (compare USSING 1943), but the relative importance of glycine is not known.

The study of the excretion of glycine in urine is hampered by the fact that no appropriate methods for the estimation of glycine in urine and plasma are known, and in the case of most other amino acids conditions are similar.

The first task in a study of amino acid excretion was therefore to work out the necessary analytical methods.

The present paper falls into three parts:

1) The description of the methods worked out for the determination of certain amino acids and groups of amino acids in plasma and urine.
2) The determination of the relative concentrations in plasma and urine of these amino acids and groups of amino acids.
3) The determination of the excretion of glycine and other amino acids after glycine ingestion.

Methods.

1. Total Amino Nitrogen.

The purpose of the experiments being a comparison of the amino acids in plasma and in urine, it was desirable to use the same method for the determinations on both media. FOLIN's (1922) colorimetric method seemed well suited for the purpose, but when used in its original form it often gives erroneous results (VAN SLYKE and KIRK, 1933). As pointed out by DANIELSSON (1933) the error is as a rule due to a blank value, which is moreover dependent on the p_H during the colour development.

A modification of the method was therefore worked out in which the error was eliminated by reading the analyses in an electric photometer, using the deflection produced by a blank as zero-point. Another deviation from the original FOLIN method is the removal of ammonia by distillation instead of the permutit treatment.

The details are the following, taking normal urine as an example: The urine is diluted 10 times; one or two ml are transferred to the distillation flask of a Parnas distillation apparatus together with 5, respectively 4 ml water. 0.5 ml 1 % Na_2CO_3 (compare FOLIN, l. c.) is added with a Krogh syringe pipette and one drop of 0.5 % phenolphthalein in 50 % alcohol is added. The vacuum distillation is commenced, the flask being heated with a micro burner. When 4 ml have been distilled off (a graduated receiver is used) the distillation is discontinued and the contents of the flask are transferred with 3 ml water into a test tube with a mark at 10 ml.; 0.5 ml n/10 HCl is added with a syringe pipette. The colour should by now match exactly with that of a blank made by mixing in a similar test tube 5 ml water, 0.5 ml 1 % Na_2CO_3, 0.5 ml n/10 HCl and one drop phenolphthalein. If the colours do not match the colour of the experimental solution is adjusted with n/10 HCl or 1 % Na_2CO_3 to the right p_H. (The mixing is preferably made by a plunger made from a slender glass tube by blowing a bulb in the lower end with a diameter about one mm less than the inner diameter of the test tubes).

1 ml freshly prepared 5 ‰ solution of sodium 1.2-naphtoquinone-4-sulfonate is now added to the unknown solutions, to the blanks and to

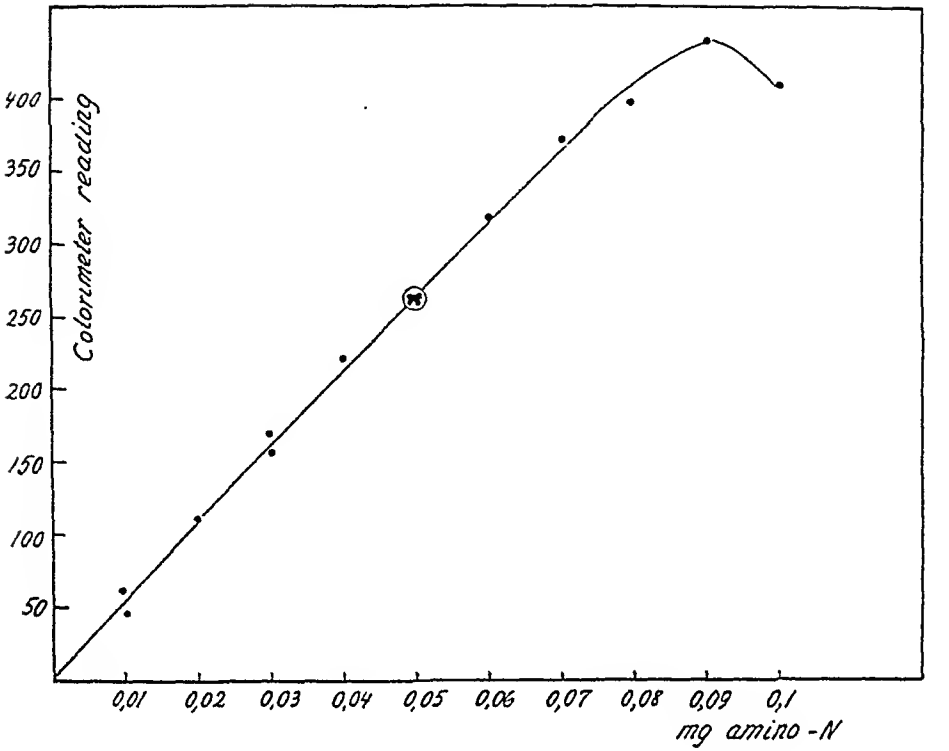


Fig. 1.

the standards and after mixing the tubes are closely corked. As a rule 0.5 ml of a solution of glycine in 0.1 n HCl is used as a standard. (Compare FOLIN l. c.). The tubes are placed in the dark for about 20 hours. Then 1 ml buffer (equal parts of 50 % acetic acid and 5 % sodium acetate) and 1 ml $Na_2S_2O_3$ (4 %) are added to each tube, water is added to the 10 ml mark and after mixing the colours are compared in an electric photometer (REHBERG, 1943) with a green filter (SCHOTT u. GENOSSEN VG9), setting the deflection produced by the blank as zero. The photometer scale is logarithmic so that the deflection measured from the chosen zero point is a direct measure of the amount of coloured substance in the solution, provided that Beer's law of dilution is valid in the present case.

Fig. 1 presents the relation between the amount of glycine amino-N used per analysis and the photometer reading. It is seen that a straight line dependency is found, when the amount of amino-N is below 0.07 mg per analysis. Above 0.09 mg amino-N the results become quite irregular, obviously because the quinone is more or less completely used up.

This is probably one of the reasons why KIRK and VAN SLYKE (l. c.) have failed to find proportionality between the amount of urine used and the colour produced, when using the original FOLIN method. If the urine is duly diluted and a correction for blank is performed, there is good proportionality between the amount of urine and the photometer-reading. This may be exemplified in fig. 2.

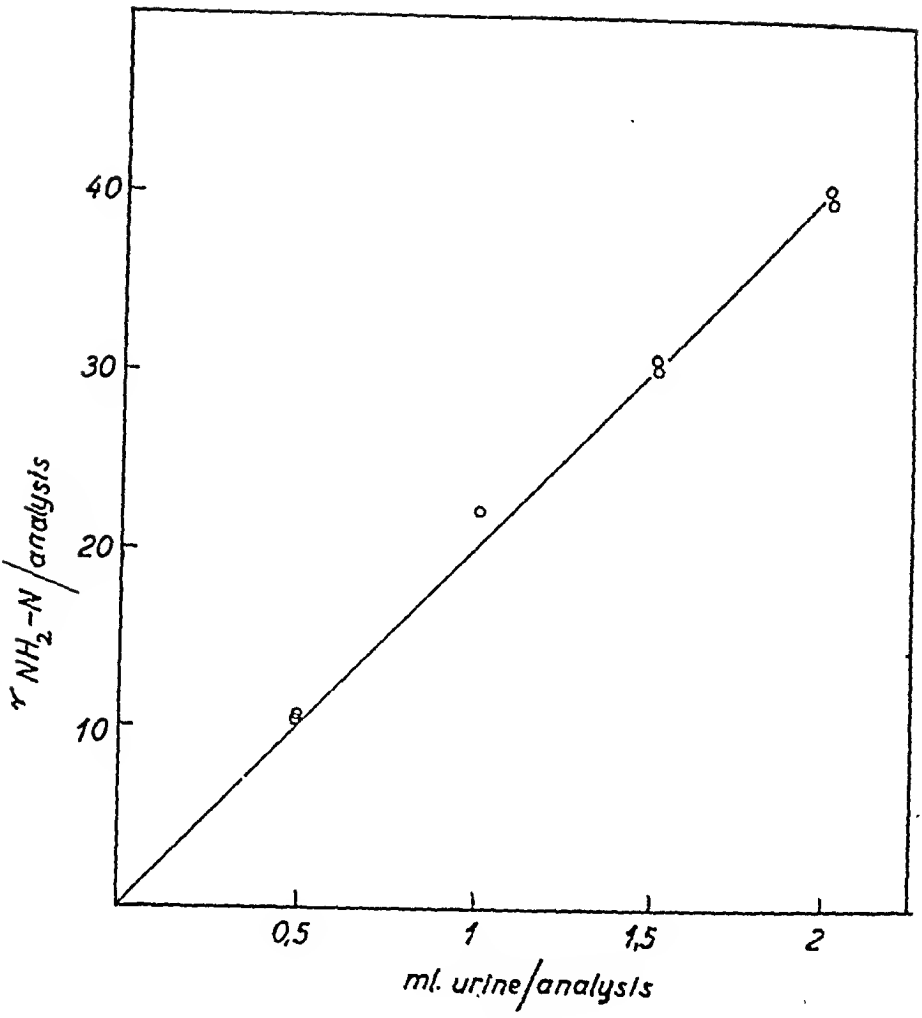


Fig. 2.

2. The Estimation of Glycine.

A truly quantitative determination of the glycine in plasma and urine is hardly possible at present. It is, however, possible to fractionate an amino acid mixture in such a way, that one fraction becomes very rich in glycine, while the other is practically free from glycine.

One way by which this aim may be reached even on dilute solutions is to make use of the great differences shown by the amino acids towards adsorptives.

Among all amino acids glycine is apparently the one which is least adsorbed for instance by carbon powder (compare TISELIUS 1941). If the difference between glycine and other amino acids were sufficiently large, it would be possible to treat a solution of amino acids repeatedly with carbon until the fall in amino-N on subsequent carbon

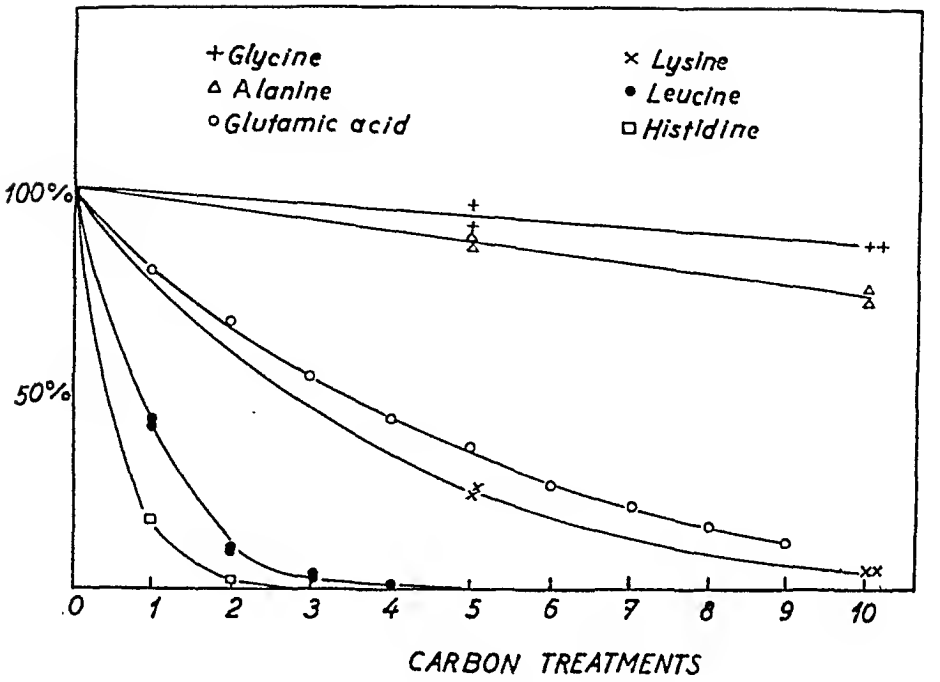


Fig. 3.

treatments corresponded to that of a glycine solution treated similarly, and then by back-extrapolation the glycine content could be estimated.

A series of experiments were made to find out if a glycine estimation in this way is possible. The amino acids tested were dissolved in 1 % NaCl and the p_H was adjusted to 7.

The amino-N concentration was as a rule chosen in the region of the amino-N concentration of the plasma, viz. 4—6 mg %. In each carbon treatment 30 mg carbon (Scherings Carbo ossium pro analysis) per ml solution was used. The results are thus independent of the absolute volume treated.

The solution was shaken with the carbon, care being taken that no carbon was floating on the surface. The solution was filtered, a sample drawn for analysis, and then the procedure was repeated until a suitable number of carbon treatments had been made.

The amino nitrogen was determined as described above; but any other suitable method may of course be used. Fig. 3 presents the results of some of these adsorption experiments. The abscissa is the number of carbon treatments, while the ordinate is the amino-N content in per cent of the starting value. Curves are drawn through the separate points; this is done for the sake of perspicuity though it is not strictly correct, because the process of adsorption is in these experiments discontinuous. Tyrosine and tryptophane are omitted in the figure, because they are practically removed after one carbon treatment. Histidine is seen to be removed on 2—3 treatments and leucine on 4—5

treatments. Glutamic acid as a representative for the acid amino acids is reduced to 10 % after 10 treatments.

The relatively low adsorption shown by this amino acid is due to the fact that the process is carried out at p_H 7, far removed from the isoelectric point (3.2) of glutamic acid. As it is well known ions are less adsorbed by carbon than are particles with no net charge. Lysine, being a rather strong base, is accordingly removed rather slowly.

Alanine and glycine are only adsorbed to a very limited extent, glycine being definitely less adsorbed than alanine.

Four carbon treatments will thus remove most monamino monocarbonic acids, whereas the slope of the curve for the 5th to 10th carbon treatment indicates if the remaining amino-N comes from pure glycine or more or less from the other weakly adsorbed amino acids.

It is necessary now to discuss briefly the relation between the absolute concentration of the amino acids in question and the relative adsorption of the same amino acids.

The simplest case would arise if the amount of any amino acid adsorbed were proportional to the concentration of that amino acid in the solution. This cannot, of course, be the case over a wider range of concentration, the carbon added having a limited adsorbing surface, from which follows a more or less pronounced tendency to saturation with adsorbed substance at higher concentration in the solution. Most adsorption processes are best described by functions like the FREUNDLICH isoterm: $y = k \cdot c^v$, where y is the amount of substance adsorbed, c is the concentration when equilibrium is reached, and k and v are constants depending on the substances concerned in the process.

But within certain limits the adsorption of the amino acids may be regarded as approximately proportional to the concentration. A few examples will show this. A solution containing amino acids was diluted four times and both the original solution and the diluted one were treated 10 times with carbon. The results are shown in table 1. It is seen that the decrease in amino-N is proportional to the starting concentration.

Table 1.

	mg% amino-N			
	Directly		After 10 carbon treatments	
	Found	Calculated	Found	Calculated
Amino acid	5.87	—	5.0	—
Same solution diluted four times ..	1.48	1.47	1.30	1.25

A histidine solution (47.5 mg % histidine) decreased to 8.3 mg % on the first carbon treatment and to 1.8 mg % on the second treatment. The decrease was thus 82 % and 79 % in the two cases although the starting values were widely different.

It thus seems permissible to regard the percentual decrease per carbon treatment as a constant between, say, 1—5 mg % amino-N.

The amount of serum filtrate available for the adsorption analysis is as a rule limited, especially when human blood is examined. The practical performance of the method was therefore adapted to relatively small samples and centrifuging was used instead of filtering. For the sake of comparison this modification was also used for the urine samples, which were moreover diluted so that the amino-N content was 2—4 mg %.

The determinations are performed as follows: 14 ml serum filtrate, neutral to lithmus is measured into an angle centrifuge tube, divided in ml's, and 14×30 mg carbon is added. After shaking and centrifuging the fluid is poured into another centrifuge tube and the calculated amount of carbon is added. This procedure is repeated until 5 carbon treatments have been made. Then a sample is drawn for analysis and the treatments are continued for further 5 times.

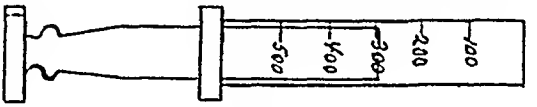


Fig. 4.

The measuring of the carbon may be facilitated by the use of a glass tube with piston shown in fig. 4. The tube is provided with marks so that the delivery of carbon may be changed from 500 mg to 50 mg. The tube is filled by aid of a nickel spatula and it is conveniently emptied when the piston is lifted out of the tube, while the mouth of the tube is turned downwards.

3. Estimation of the Sum of Leucine and Valine.

In a previous paper (USSING, l. c.) a micro modification of FROMAGEOT's method was described which enabled the estimation of the sum of leucine and valine in one g's samples of blood and tissues. (The amino acids were deaminized and the resulting hydroxy acids were oxydized with chromic acid, only the two amino acids mentioned form acetone.) These amino acids were of special interest in the present investigation on account of their lack of active groups other than the amino group and the carboxylic group. Therefore, it would seem probable that they were reabsorbed by the same mechanism as is glycine.

In the form described the method could not, however, be used on urine, because the urea would decompose about 10 times the nitrite normally used for the deamination process. It was therefore decided to separate the amino acids in question from urea. This proved to be possible by adsorption of the amino acids in 70 % alcohol solution on Al_2O_3 according to WIELAND (1942 a). Under these conditions urea is not adsorbed; but urine contains other substances which destroy nitrite and which are held back by Al_2O_3, and it was therefore necessary to increase somewhat the amount of nitrite used.

The analysis is performed as follows: The urine is made acid to congo-paper with concentrated hydrochloric acid and is then extracted for one hour with ether in a continuous extraction apparatus in order to remove β-hydroxy-butyric acid and other substances which on oxydation might give acetone. The ether is removed by aeration, the solution is neutralized and an amount corresponding to 4 ml of the original urine is transfeired to a cylinder and 96 % alcohol is added to the 15 ml mark. An aluminium oxide column is prepared from 5 g Al_2O_3 (Merck) suspended in 10 ml 70 % alcohol in the apparatus described by WIELAND (1942 b). The urine containing solution and after that 10 ml 70 % alcohol is sucked through the column. When the column has been sucked practically dry, the centrifuge tube acting as a receiver is emptied and replaced and then 40 ml water is sucked through the column. The amino acids will then be almost quantitatively eluated. The watery solution, which has become alkaline during the passage through the Na^+-containing Al_2O_3-column, is neutralized and evaporated to dryness in vacuo.

The residue is taken up in 10 ml water. From the resulting solution 5 ml is transferred to the pyrex tube in which the deamination is performed. The procedure is that described by USSING (l. c.) with the exception that the amounts both of n-sulfuric acid and of 2.5 % sodium nitrite are tripled. From this point the procedure is identical with that described previously, including concentration of the solution by distillation, oxydation with CrO_3 in acetic acid in sealed ampoules, distillation of the acetone formed and colour development in Conway units.

Table 2.

	mg leucine + valine found (calculated as leucine)			Recovery %
	1 ml urine	1 ml leucine solution	1 ml leucine solution + 1 ml urine	
Exp. I	0.029	0.470	0.489	99
	0.029	0.493	0.523	
Exp. II	0.048	0.470	0.492	94
	0.055	0.493	0.507	

Table 2 shows the results of some control experiments. It is seen that nearly all leucine added to urine is recovered. As we have no valine available only control experiments with leucine have been performed.

4. The Determination of Histidine.

After different methods had been tried it was found that the method of RACKER (1940) was well suited for the determination of histidine in urine. Compared with KAPPELER-ADLER's method (1933) it has the advantage that it is less sensitive to disturbing substances. Moreover

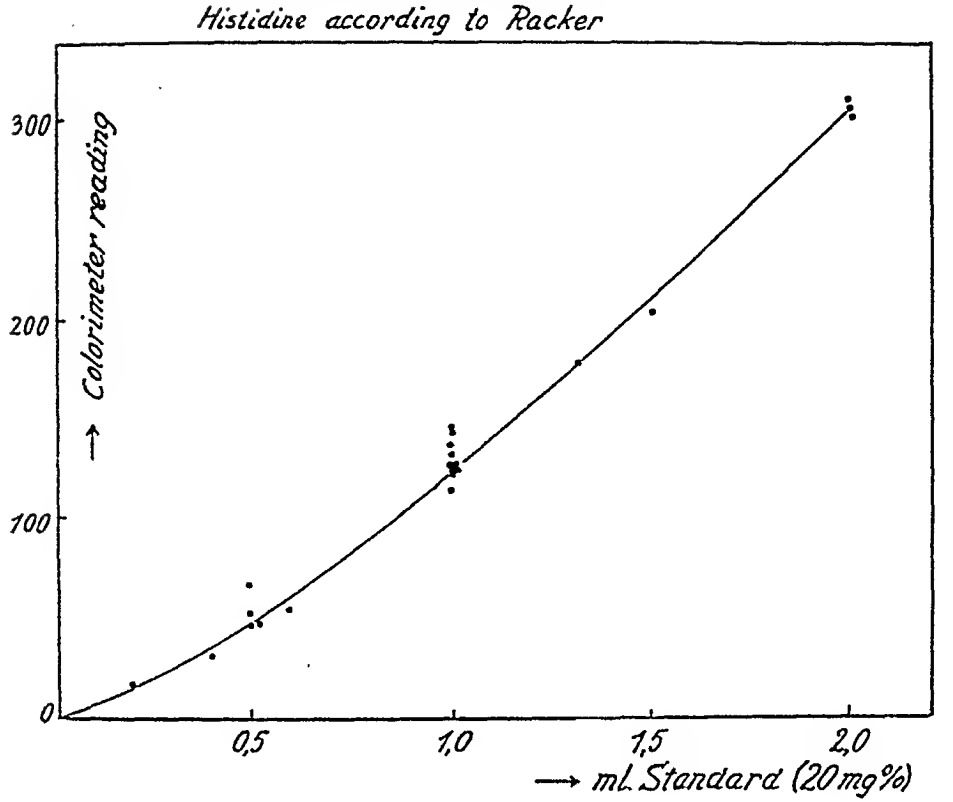


Fig. 5.

the KAPPELER-ADLER method gives unduly low colour values for low concentrations of histidine.

This deviation from proportionality between histidine concentration and coloured substance is also shown by the RACKER method, but to a lesser extent. Fig. 5 shows the relation between histidine concentration and colorimeter reading in arbitrary units when a green filter is used. This curve has been used for the calculation of the results presented in the following.

In the original RACKER method histidine is determined directly on the urine. In certain cases, especially when high concentrations of other amino acids are expected in the urine it would be preferable to isolate the histidine before the determination because some amino acids, for instance tyrosine, give a similar though less intense colour, while other amino acids like glycine may in high concentrations affect the colour-development adversely (compare table 3).

As stated above histidine is strongly adsorbed on carbon. On the other hand it was found that histidine is easily eluated again from adsorption on carbon by 50 % acetic acid.

These facts may be used in a partial separation of histidine from other amino acids. The following procedure has been used: 15 ml urine is neutralized to lithmus and one g carbon powder is added. After thorough

shaking the suspension is filtered on a BÜCKNER funnel and the carbon layer is washed with a few ml water. The flask is emptied and washed and then 15 ml 50 % acetic acid is sucked through the carbon layer. The acetic acid, now containing the histidine is concentrated to a syrup in vacuo. The residue is taken up in 15 ml water so that each ml corresponds to one ml original urine. In the subsequent histidine determination according to RACKER, the precipitation of phosphates with $Ba(OH)_2$ is omitted, because the solution prepared in the above way is practically free from phosphate.

Table 3.

	Colorimeter reading
1 ml histidine standard	140
	140
» » » » +2 mg glycine	136
	134
» » » » +4 » »	136
	141
» » » » +8 » »	100
	125

Before reading in the photometer all analyses are centrifuged to remove a slight turbidity.

This method of partial histidine isolation has as its first aim to get rid of glycine, which may occur in the urine in an appreciable concentration when it is given by mouth. Moreover the greater part of the tyrosine is removed because this amino acid is not readily eluated with 50 % acetic acid. This is exemplified in table 4. It is seen that about 70 % of added tyrosine is lost when the above histidine isolation method is used.

Table 4.

25 ml urine + 5 ml tyrosine standard (15 mg% tyrosine) adsorbed on 2 g carbon. Eluation with 30 ml 50 % acetic acid. Eluate concentrated to dryness and taken up in 10 ml water. One ml, corresponding to 0.5 ml tyrosine standard + 2.5 ml urine, taken for analysis. 25 ml urine+5 ml water treated in the same way.

	mg tyrosine found	% recovery found tyrosine
0.5 ml tyrosine standard	0.075	—
urine+water	0.074	—
urine+tyrosine	0.098	32

As seen from fig. 3 histidine is not entirely removed after one carbon treatment, about 17 % being left in the solution. Although in the isolation experiments presented here the double amount of carbon is used, it is reasonable to assume that about 10 % of the histidine is lost. Recovery experiments with added histidine would of course have been of value, but have to be postponed till after the war due to lack of histidine. As the error in the histidine determination according to RACKER may sometimes exceed 10 %, the question about the exact

recovery is at present of minor importance. Table VIII shows examples of histidine determinations directly according to RACKER and after partial isolation as described above. The results show a satisfactory agreement.

The Relative Concentration of Different Amino Acids in Plasma and Urine.

As stated in the introduction it is necessary to know something about the relative concentration of the different amino acids in the urine and in the plasma, before amino acid ingestion experiments are performed. Therefore some experiments were done in which blood and urine were taken simultaneously from the experimental subjects. Analyses on urine were made as described in the foregoing.

The blood, about 40 ml, was collected in a centrifuge tube and immediately centrifuged. After a quarter of an hour the coagulum was loosened from the sides of the vessel and the centrifuging was continued until the coagulum had contracted. The serum was measured in a cylinder, 3 vol. of water and 1 vol. of 20 % trichloroacetic acid were mixed with the serum and the mixture was left at room temperature for one hour. Then further 5 vols of water were added so that the total dilution became 10 times the original amount of serum. The precipitate was filtered off and the filtrate was measured. A few drops of concentrated hydrochlorie acid were added and the excess trichloroacetic acid was removed by extraction with ether for one hour in a continuous extractor. After neutralisation the solution was concentrated to a small volume in vacuo and then transferred to a cylinder and adjusted so that one ml corresponded to one ml of the original serum. On this solution the analyses were performed.

The results are presented in tables 5 and 6. Table 5 shows the actual concentrations found, whereas table 6 shows the concentration indices which indicate how many times the different substances are concentrated in the urine as compared with the plasma.

Turning first to table 5 it is seen that the amino-N of the urine decreases very markedly on 10 carbon treatments. This is very important because it means that glycine is not the all dominating amino acid in the urine as was suggested by ABDERHALDEN and SCHITTENHELM (1906). The amount of glycine may be less, but not much higher than the amount indicated by the amino-N values after 10 carbon treatments.

Table 5.

Amino acids in urine and in serum.

Subject		Amino-N mg%		Leucine + Valine mg%	Histidine mg%	Tyrosine mg%
		Total	After 10 carbon treatm.			
L.....	Urine	16.3 16.0	7.0 6.8	—	—	—
	Serum	5.7 5.4	1.75 1.73	—	—	—
C.....	Urine	16.7 15.6	4.9 4.6	14	—	—
	Serum	5.4 5.1	1.30 1.33	10	—	—
L. J.	Urine	15.3 13.9	4.3 4.3	13.5	—	—
	Serum	6.5 6.8	1.59 1.59	10.6	—	—
B.....	Urine	18.0 17.0	5.6 5.8	3.0	18.6 17.8	ca. 10
	Serum	5.4 5.3	0.96 0.92	6.7	ca 6	2

Similarly only a minor part of the amino-N of the serum comes from glycine. Leucine + valine (calculated as leucine) is remarkably low in the urine compared with the amount of these amino acids in serum. Whereas the concentration of total amino-N and of amino-N after 10 carbon treatments is much higher in the urine than in the plasma in all cases, this is not the case with leucine and valine. In one case the concentration of these amino acids is even higher in plasma than in urine. As to histidine the value found for blood is probably somewhat high. During the colour development a blue colour appeared first which later vanished, or it was possibly covered by the orange red colour coming from histidine. This blue colour indicates the presence in the blood of a substance which also gives a colour reaction under the conditions of the analysis. The blue colour was repeatedly seen when analyses on serum filtrates were performed. An attempt was made to isolate histidine from ox serum filtrate by the carbon treatment method, but no histidine could be detected although the direct determination gave 3 mg % histidine.

Table 6 shows that the amino acids left after 10 carbon treatments have a somewhat higher concentration index than have

the bulk of amino acids. Leucine + valine on the other hand have a definitely lower concentration index. Histidine seems to behave like the bulk of amino acids, but as mentioned the serum value for histidine may be too high, which means that the concentration index calculated may be too low.

Table 6.

Concentration index of different amino acids.

Subject	Total amino acid	Amino acid after 10 carbon treatm.	Leucine + Valine	Histidine	Tyrosine
L.....	2.9	3.96	—	—	—
C.....	3.05	3.6	1.4	—	—
L. J.....	2.2	2.72	1.3	—	—
B.....	3.28	6.1	0.45	3	ca. 5

It is obvious from the data presented here that the amino acids are not treated equally by the reabsorption mechanism of the kidney, but it is left undecided whether the amino acids are reabsorbed by one mechanism which prefers leucine and valine for glycine or whether the different amino acids are reabsorbed by more or less independent mechanisms. It may be assumed, however, that if one mechanism is responsible for the reabsorption of all amino acids it would be difficult to saturate the mechanism with glycine to such an extent that leucine and valine were excreted in abundance whereas a definite histidine excretion might result from a suitable saturation of the reabsorption mechanism with glycine.

The Amino Acids Excreted on Glycine Intake.

The subjects were fasting, but were allowed to drink a cup of tea two hours before the beginning of the experiment. One hour before the taking of glycine the bladder was emptied, and the urine formed in the last hour was used for the control determinations. 20 g of glycine or, in one experiment, 25 g were given in 150 ml water and the urine was voided after one hour and again after 2 and in some experiments after 3 hours. In the first two experiments the urine from the first one hour period was used for the analyses, but as it was found that the maximum excretion

of amino-N was reached in the second hour, determinations were made on the urine from this period and on the urine from the third one hour period as well.

Table 7.

		Urine sample ml	Total amino-N mg%	Amino-N after 5 carbon treatm.	Amino-N after 10 carbon treatm.	Histidine mg%	
						Directly	After isolation
I.	Last one hour period before taking 20 g glycine	50	19	—	11.5	25.2 26.2	—
	First one hour period after glycine intake	37	63	—	58	22.0 28.2	—
II.	Last one hour period before taking 20 g glycine	50	21.5 20.8	12.7 13.5	11.7 10.9	—	19.4 19.8
	First one hour period after glycine intake	47	66.3 68.2	55.7 52.9	47.6 45.6	—	18.8 18.6
	Second one hour period after glycine intake	60	158 154	—	—	—	—

The results are shown in tables 7, 8 and 9. Taking table 7 first, it is seen that, whereas the excretion of total amino-N is more than tripled after the glycine intake, the histidine excretion is not affected. In the next experiment (table 8) similar results were found for the first one hour period. In the second period the amino-N increases still further and is now 9 times the normal value, but still the histidine excretion is unaffected. The last column shows the excretion of tyrosine as determined by ARNOW's modification of the Millon reaction (ARNOW, 1937) on ether extracted urine. The values are not corrected for the blank coming from the urine pigments. This blank is about one third of the value; but even so it is obvious that the excretion of tyrosine is practically unaffected by the excessive amino acid excretion produced by glycine. The determinations of amino-N after 5 and 10 carbon treatments show, that whereas glycine, or amino acids adsorbed equally little as this amino acid, play a minor part in the control period, this group of amino acids dominate after the glycine intake. Indeed all the increase in amino-N seems to originate from glycine. This may be shown by a back calculation from the

Table 8.

	Urine sample ml	Total amino-N mg%		Amino-N mg% after 5 carbon treatm.		Amino-N mg% after 10 carbon treatm.	Histidine mg%		Tyrosine mg% not corrected for blank
		Found	Calcul.[1]	Found	Calcul.[1]		Directly	After isol.	
Last one hour period before taking 20 g glycine	78	20.4	8.7	11.2	8.1	7.5	18 17.8	20 19	15
First one hour period after glycine intake	55	75.4 76.4	75	73.0	70	64.9	19.6	18.5 18.0	12.5
Second one hour period after glycine intake	62	175 185	173 181	165 167	160 168	149 156	17.0	18.5 17.2	10
Third one hour period after glycine intake		159 157					18.0		

amino-N after 10 carbon treatments, assuming that the slope of the adsorption-curve is that of glycine (see fig. 3). It is seen that the values found for amino-N after 5 carbon treatments and before the treatments are very nearly identical with the values calculated. Even when due allowance is made for experimental error it is clear that nearly all amino-N excreted after glycine intake comes from glycine.

A similar experiment is presented in table 9. The subject was given 25 g glycine in 200 ml water. In this experiment the diureses varied more than in the other experiments. It is seen that the concentration of histidine is constantly falling, but at the same time the amount of urine excreted per hour is increasing, so that the histidine excretion per hour is nearly unaffected by the increase in amino acid excretion following glycine intake. The amino-N excretion reaches its peak in the second hour period after glycine intake. From the rate of decrease in amino-N during carbon treatment it is concluded, just as in the foregoing experiment, that glycine only is excreted (in excess) when this amino acid is given to the subjects.

[1] Calculated on the basis of amino-N after 10 carbon treatments, under the assumption that all amino-N originates from glycine.

Table 9.

	Urine voided ml	Total Amino-N mg%		Amino-N mg% after 5 carbon treatm.		Amino-N mg% after 10 carbon treatm.	Histidine mg% directly
		Found	Calcul.	Found	Calcul.		
Urine, last one hour period before taking 25 g glycine	40	21.8 23.0	11.6	11.9 12.3	10.6	10.3 9.7	25.8
1' one hour period after glycine intake	45	106.3 105.3	98.5	97.5 90.8	91	83.5 86.5	23.6
2' one hour period after glycine in. take	64	280 280	295	270 271	274	257 253	21.0
3' one hour period after glycine intako	85	141 153	145	136	134	124 126	14.0
Serum, 2 hours after glycine intako		10.3 10.42	7.74	7.35 7.64	7.18	6.68 6.68	

In this experiment blood samples were taken before and two hours after the glycine intake. The first sample was, however, lost; but the analyses in table 5 (L) are from the same person so that 5.6 mg % amino-N in serum may be taken as the fasting value. The analyses on the serum from the blood sample taken 2 hours after glycine ingestion (bottom of table 9) show that the amino-N has increased to 10.4 mg %. KIRK (l. c.) found a similar increase, when patients were given 25 g glycine.

Discussion.

Although large quantities of glycine are excreted when the glycine content of the blood is raised, only a part of the glycine filtered out in the glomeruli can be accounted for in the urine. Filtration determinations were not made in the experiments presented here; but, as SMITH (1937) puts the average filtration in man at some 120 ml per minute, 100 ml per minute is a conservative estimate. The excess amino-N in serum two hours after glycine ingestion was found to be about 5 mg % (compare table 9). The excess filtration of amino-N per hour is thus 100×

$\times 60 \times \frac{5}{100} = 300$ mg. In the second one hour period after glycine ingestion in the same experiment the amino-N content of the urine was 280 mg % of which some 260 mg % may be regarded as excess excretion. The volume of urine from this period was 64 ml. Thus the excretion was $260 \times \frac{64}{100} = 166$ mg; while 300 mg amino-N have filtered out, only 166 mg have been excreted. This calculation is of course very approximative, but if the figures are in error, the true values are likely to give a greater difference between the amino-N filtered out and the amount excreted. At first sight this seems to indicate a rather efficient reabsorption of glycine even when considerable amounts of this amino acid are excreted; but the apparent reabsorption of nearly half the filtered glycine from urine containing many times the normal amount of amino-N is probably best explained as a back diffusion, just as the major part of the urea diffuses back to the blood according to REHBERG (1926).

The reabsorption at normal plasma amino-N concentration cannot be explained as the result of diffusion. Diffusion is proportional to the concentration difference, whereas the experiments of KIRK (l. c.) and the results presented here show that the amino-N clearance increases with increasing serum amino-N.

Thus at least two factors seem to be of importance for the reabsorption of glycine: 1) back-diffusion which sets in when the amino-N concentration in the tubuli becomes higher than in the blood, and 2) either a specific mechanism which is only active when the glycine concentration is below a certain level, or, in analogy with the reabsorption of other treshold substances, a mechanism which can only transfer a certain amount of glycine per unit time.

The experiments described in the foregoing chapter seem to indicate that glycine is not reabsorbed by the same mechanism as are the other amino acids. This result leads to the question if there are individual reabsorption mechanisms for each of the more than twenty known amino acids.

A closer discussion of this problem may seem premature until the excretion conditions of more amino acids are known. It may, however, be permissible to point out a possibility which would make the assumption of the many individual mechanisms easier to accept.

If the tubule cells were able to synthesize protein at a fairly high rate from the amino acids in the lumen of the tubuli, then the amino acids would be reabsorbed in relation to their molecular ratios in the assumed protein. Any amino acid being in excess would on the other hand be excreted. Thus it could be explained that the ingestion of large amounts of glycine leads to the excretion of that amino acid only. The protein formed might then be split into amino acids again in the abluminar part of the cells. The study of protein formation in the latest years by the aid of isotopes (SCHOENHEINER, RATNER and RITTENBERG, 1939, USSING, 1941) and ultraviolet-absorption (CASPERSSON's school, see for instance HYDÉN, 1943) have revealed that protein renewal may proceed at an astonishingly high rate, so that quantitatively the supposed reabsorption mechanism might well be able to work.

Summary.

1. Methods are described for the estimation of total amino-N, glycine, leucine + valine and histidine in blood plasma and urine. The amino-N determination method is a modification of FOLIN's method. Glycine is estimated from the fall in amino-N on repeated adsorptions on carbon.

For the determination of leucine + valine in urine a method is described modifying a method previously worked out by the author for the determination of the sum of these amino acids in small tissue samples.

Histidine is partly isolated from urine by adsorption on carbon and eluation with 50 % acetic acid.

2. The content of different amino acids in plasma and urine from human subjects is examined. It is found that glycine is not the all dominating amino acid, neither in plasma nor in urine.

Moreover it is found that the concentration index for glycine (possibly + other little adsorbed amino acids) is somewhat higher than that for total amino-N. Leucine and valine are very efficiently reabsorbed compared with for instance glycine and histidine.

3. The effect of glycine ingestion in human subjects is examined. It is found that the taking of 20—25 g glycine may increase the amino acid content in the urine up to 14 times. The excess excretion is, however, shown to consist of glycine only, the excretion of histidine and tyrosine remaining at the fasting level.

4. The results are discussed and a working hypothesis is set forth to explain the apparent independent reabsorption of glycine and other amino acids.

The author is greatly indepted to Professor KROGH and to Dr. REHBERG for the interest they have shown in my work. I likewise wish to thank Mrs. K. CHRISTIANSEN, who has made many of the analyses.

Literature.

ABDERHALDEN, E. in A. SCHITTENHELM, Hoppe Seyl. Z. 1906. *47.* 339.
ABDERHALDEN, E. and E. TETZNER, Ibidem. 1935. *232.* 79.
ARNOW, L. E., J. Biol. Chem. 1937. *118.* 531.
DANIELSON, I. S., Ibidem 1933. *101.* 505.
DOTY, J. R., Proc. Soc. exp. Biol. N. Y., 1941. *46.* 129.
DOTY, J. R. and EATON, A. G., J. Nutrit. 1939. *17.* 497.
FOLIN, O., J. Biol. Chem. 1922. *51.* 377.
HYDÉN, H., Acta Physiol. Scand., 1943. *6.* Suppl. 17. Diss.
KAPPELER-ADLER, R., Biochem. Z. 1933. *264.* 131.
KIRK, E., Acta med. Scand. 1936. *89.* 450.
KREBS, H. A., Biochem. J. 1935. *29.* 1620.
MEDES, G., Ibidem 1937. *31.* 12.
RACKER, E., Ibidem 1940. *34.* 89.
REHBERG, P. BRANDT, Ibidem 1926. *20.* 461.
REHBERG, P. BRANDT, Acta Physiol. Scand. 1943. *5.* 277.
SCHOENHEINER, R., I. RATNER and D. RITTENBERG, J. Biol. Chem. 1939. *130.* 703.
SMITH, HOMER W., The physiology of the kidney, London. 1937.
TISELIUS, A., Ark. Kemi, Mineral. Geologi, 1941. *15.* No. 6. 1.
USSING, H. H., Acta Physiol. Scand. 1941. *2.* 209.
USSING, H. H., Ibid. 1943. *6.* 222.
VAN SLYKE, D. D. and E. KIRK, J. Biol. Chem. 1933. *102.* 651.
WIELAND, T., Naturwiss. 1942 a. *30.* 374.
WIELAND, T., Hoppe-Seyl. Z. 1942 b. *273.* 24.

From the Laboratory of Zoophysiology, Copenhagen University.

The Determination of Chloroform in Tissues and Blood.

By

HANS H. USSING.

Received 27 October 1944.

In the course of an investigation of the permeability of brain capillaries to different substances which is being carried out in this laboratory, a method was required for the determination of minute amounts of chlorofom in tissues and blood. The demands to a suitable method were the following: 1) Concentrations ranging from 0.1 mg/g to 1 mg/g were to be determined within a probable error of no more than 5 %. 2) The samples of tissue should not exceed 1 g or in some cases 0.5 g.

All attempts to adopt the wellknown method of NICLOUX (1927) for the purpose were unsuccessful, but at last we succeeded in making a modification of the method described by COLE (1926) which could fulfil the above requirements. Use is made of a coloured substance which is formed when chloroform is heated with pyridine in strongly alkaline solution.

In COLE's performance of the method, the coloured pyridine layer, which is formed on account of the immiscibility of pyridine and strong alkali, is pipetted off and compared with a standard colour.

It is impossible to make this comparison in a photometer, since the pyridine layer is opaque from a content of minute droplets from the underlying watery solution. This was one of the reasons why the original method, despite being very sensitive, was not directly suitable for our purpose.

As the mutual solubility of two substances is as a rule aug-

mented by the addition of a third substance which is miscible with both, we added ethyl alcohol to the opaque pyridine which contained the coloured substance, and the result seemed at first satisfactory as the solution became quite clear. The results were, however, subjected to irregular deviations; the reason proved to be that the coloured substance acted as a pH indicator, only showing the maximum colour in the extreme alkaline region. Even the CO_2 from the air sufficed to fade the colour, and breathing directly into the test tube containing the pyridine-alcohol mixture eventually made the colour disappear.

Therefore alkaline alcohol was introduced for clearing the pyridine layer and since then it has been possible to make multiple determinations on known solutions with small deviations only (see table 1).

Table 1.

Colorimeter reading.

6 samples of 0.4 ml chloroform standard	Determinations on 7 samples of the same chloroform-containing blood
170	190
170	194
170	190
161	190
173	190
170	193
	191

It should be recommended, however, to make the readings in the photometer within 5—10 minutes after diluting the pyridine phase, as the coloured substance is not quite stable over a longer period.

As the method was to be used on blood as well as on tissue samples it was impossible to use COLE's technique, viz. extraction with acidified water. A distillation method had to be worked out which would allow the quantitative recovery of the chloroform content of the sample. In accordance with the poor solubility of chloroform in water, the vapour tension of chloroform when dissolved in water is extraordinarily high. It is obvious that the loss of chloroform due to evaporation to the air during the distillation, would be much reduced if the chloroform vapour were trapped in a liquid with a higher dissolving power towards chloroform.

To obtain such conditions the narrow tip of the neck of the distillation apparatus is ending under a measured amount of pyridine in the graduated test tube serving as a receiver. The test tube is placed in an ice bath to further diminish the loss by evaporation.

The distillation is performed as a steam distillation. This method proved to be preferable to direct heating on the distillation flask, which sometimes produced volatile coloured substances due to decomposition of organic matter.

When blood or tissue samples are distilled, a suitable amount of sodium tungstate and sulfuric acid is added in order to precipitate the protein and so prevent foaming. The mixture of Na-tungstate and sulfuric acid is so adjusted as to give a faintly acid solution, which prevents a possible decomposition of chloroform.

Performance of the Analysis.

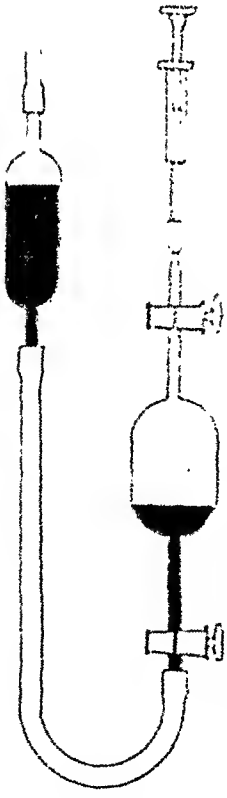


Fig. 1.

The reagents used are the following:

Potassium hydroxyde 60 % in water
Pyridine (or "pyridine base")
96 % alcohol
25 vol% sulfuric acid
10 % sodium tungstate
chloroform standard solution (see below).

Pure pyridine may be used; but due to the war conditions we had only a very limited amount of this substance available.

In practically all experiments "pyridine base", a mixture of pyridine homologues with at most 50 % pyridine content, was used and apparently the colour develops just as well with this mixture as with pure pyridine.

The chloroform standard solution is kept in a gas sampling vessel over mercury (see fig. 1) so that there is no air space over the solution. Samples are drawn through the cock in the top of the vessel with a KROGH's syringe pipette (KROGH, 1935), which fits in a short rubber tube on the outlet of the cock.

The standard solution is prepared as follows: 50 ml water (previously freed from air by boiling) is introduced into the vessel followed by

45 mm³ pure chloroform delivered by a KROGH syringe pipette with a tip long and narrow enough to reach through the bore of the cock; after this another 50 ml water is taken in and then the cock is closed. (During the whole procedure no air should be allowed to enter; the water is slowly sucked in by lowering the levelling bulb shown in the figure.) The lower cock is closed too, the vessel is disconnected from the levelling bulb, shaken vigorously and allowed to stand over night so that the small amounts of chloroform adhering to the walls of the vessel may dissolve.

The vessel is now connected again with the levelling bulb and is ready for use. Kept in the dark this standard, containing 0.66 mg chloroform per ml, is stable for months.

1. Determination of Chloroform in Blood.

One ml blood (less if the content of chloroform is expected to be above 0.05 mg) is measured into the distillation flask I (fig. 2), using a KROGH's syringe pipette. The flask which should beforehand contain 4 ml water, 1 ml sodium tungstate solution and 0.2 ml 25 % sulfuric acid, is now quickly connected with the distillation apparatus. A test tube with a mark at 6 ml and containing 1 ml pyridine serves as a receiver (if a high content of chloroform is expected 2 ml pyridine may be used).

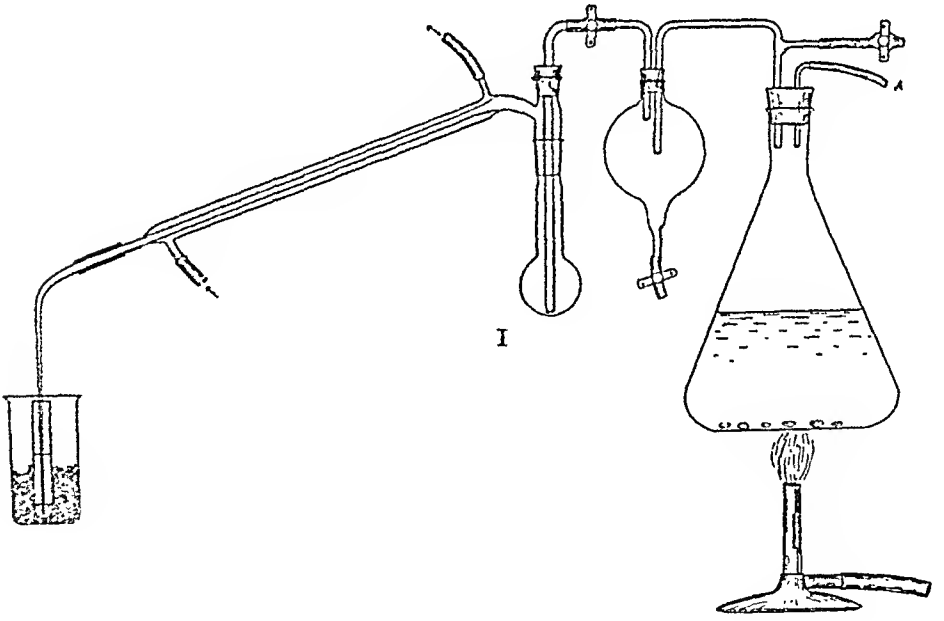


Fig. 2.

The cock between the distillation flask and the steam generator is opened and the distillation is continued until the 6 ml mark on the receiver is reached.

Through the tube A air under moderate pressure may be let in at intervals if a tendency of sucking back from the receiver to the distillation flask should develop. If even boiling is maintained in the generator, such precautions will as a rule be unnecessary.

Two ml 60 % KOH is now added to the distillate and the contents of the tube are very thoroughly mixed.

A known amount of chloroform standard solution is measured into a similar tube, also containing 1 ml pyridine. Water is added to the 6 ml mark and KOH is added as above. The tubes, loosely corked, are heated 1.5 minutes in a boiling water bath. After cooling 0.5 ml of the supernatant pyridine phase is measured into a small test tube and diluted with 4 ml alcohol and 0.5 ml 60 % KOH. After mixing the solutions are compared in the photometer, using a green filter.

2. Determinations on Tissue Samples.

Two methods have been used, one being suited for all tissues while the second is specially suited for brain tissue for which on the other hand it gives very reliable results.

First method: The tissue samples are immediately frozen in dry ice and kept so until they are to be analyzed. A suitable amount (about one g) tissue is pulverized in a dry ice cooled mortar; the powder is transferred into a weighed distillation flask containing 4 ml water and rapidly weighed again. After this the procedure is identical with that described for blood. To be sure that no chloroform is left in the tissue it is advisable to continue the distillation into a fresh receiver with pyridine when the mark has been reached in the first one. When the mark of the second receiver has been reached the distillation may be stopped. A third distillate seems never to give any colour.

Second method: The brain is frozen *in situ* with dry ice. Suitable samples are excised and transferred directly to the interior of a cooled injection syringe from which the piston has been pulled out. Then the piston is replaced and pushed in until most of the air has been expelled. The contents of the syringe is allowed to thaw, the syringe is weighed with the cannula put on. (The cannula is 10 cm long and with a 1 mm bore.) It is now possible

without unduly hard pressure, to expel a suitable amount of the brain through the cannula directly into the distillation flask upon which the procedure is identieal with that used for blood. The syringe is weighed again in order to determine the amount analysed. Several determinations may be made from the contents of one syringe.

Discussion.

As seen from table 1 the results can be well reproduced. Table 2 gives a comparison between samples of chloroform standard

Table 2.

	Determined directly colorimeter reading	Distilled from 1 ml blood colorimeter reading
0.2 ml chloroform standard	112	108
		113
	114	123
0.4 ml chloroform standard	230	223
	226	

solution determined directly and distilled from a solution with added blood. It is seen that no loss takes place during distillation. Moreover it is seen that the colour intensity (expressed in the photometer reading in a logarithmic scale, compare REHBERG (1943)), is proportional to the amount of chloroform in the sample. Table 3 shows some determinations made on tissues

Table 3.

	Chloroform mg/g tissue
Brain, powdered in dry ice cooled mortar	0.152
	0.156
Same brain minced at room temperature	0.106
Liver, powdered in dry ice cooled mortar	0.764
	0.766
Same liver, minced at room temperature	0.556
	0.485

from a rabbit which had got an intravenous injection of a chloroform solution, pulverized in the frozen state and minced quickly at room temperature; it is seen that considerable losses occur if vigorous cooling is omitted.

Table 4.

	Sample g	1′ dist. reading	2′ dist. reading	3′ dist. reading	Total reading	0.2 ml chlorof. standard reading	mg chloro-form in sample	mg chloro-form pr. g tissue
Guinea-pig-brain	0.482	73	10	2	85	112	0.095	0.206
	0.305	45	6	0	51	114	0.060	0.196
Cat-brain ...	0.640	30	8	1	39	104	0.049	0.077
	0.534	33	0	0	33	104	0.042	0.078
Cat-brain ...	0.732	39	6	0	45	90	0.068	0.091
	0.695	36	3	0	39	87	0.058	0.083
	0.670	42	2	0	44	87	0.065	0.097

Table 4 shows some determinations on chloroform containing brain from a guinea-pig and two eats, performed aeeording to the seeond method mentioned above (the brain is squeezed out into the distillation flask from an injeetion syringe). It is seen that good aecordance is obtained between duplieate determinations and the losses due to evaporation are bound to be low. Moreover it is improbable that any chloroform is held baek in the brain tissue as the third distillate is practieally free from ehloroform. This teehnique is probably the best way to get reliable determinations on brain; most other tissues are, however, too rigid to allow the squeezing-out procedure.

Summary.

Cole's chloroform determination method is modified so as to permit the use of an electric photometer. A distillation technique is deseribed which permits the quantitative determination of ehloroform in blood and tissues, especially brain tissue.

I wish to thank Professor Krogh for the constant interest he has taken in my work. I am likewise indebted to Mrs. I. Lundquist and Mrs. K. Christiansen who have performed most of the analyses.

References.

Cole, W. H., J. biol. Chem. 1926. *71*. 173.
Krogh, A., Industr. Eng. Chem. 1935. *7*. 130.
Nicloux, M., C. R. Soc. Biol. Paris 1927. *97*. 1320.
Rehberg, P. Brandt, Acta Physiol. Scand. 1943. *5*. 270.

From the Department of Medical Chemistry, University of Uppsala, Uppsala.

Studies on the Action of the Thiamin Destroying Fish Factor.

By

GUNNAR ÅGREN.

Received 28 October 1944.

After GREEN, CARLSSON and EVANS (1941) had discovered that a disease of foxes, caused by feeding fish, could be cured by thiamin, it was of interest to investigate in what manner this inactivation was accomplished. There were several possibilities. The thiamin could be enzymatically inactivated, or combine with a specific protein in a manner similar to the inactivation of biotin by avidin (EAKIN et al. 1941). It may also be significant that ROSE in 1938 reported that animals deprived of valine exhibited unusual symptoms, including sensitiveness to touch and absence of muscular co-ordination, symptoms which are also exhibited by foxes suffering from Chasteks paralysis. Through the investigations of SEALOCK et al. (1943) and KRAMPITZ and WOLLEY (1944) it was shown that thiamin was inactivated by enzymes present in the viscera of carp. The latter authors also demonstrated that, in the reaction, thiamin was split up into the pyrimidine and thiazole halves. Recent researches from this laboratory demonstrated the presence of rather high concentrations of valine in fish protein (ÅGREN, 1944). The thiamin inactivating factor has been found in 11 of 30 investigated Swedish fishes (LIECK and ÅGREN, 1944). In the latter paper the enzymatical reaction was also studied. It was demonstrated that the inactivation of the thiamin molecule was probably not effected by an oxidative process. In the present paper, interest was concentrated on reductive inactivations of thiamin.

Experimental. The thiamin-inactivating fish factor was extracted from viscera of carp, crucian, ide and rudd. The viscera were collected using the same precautions as previously described, and were stored at — 15° (LIECK and ÅGREN). Thiamin was determined by the method of MELNICK and FIELD (1938), as described in the previous paper. It was assumed that no real inactivation had taken place unless the extinction values of the digestion tests were more than 20 per cent lower than the control tests. When not otherwise stated 1 ml aliquots of thiamin solutions containing 700 γ of vitamin were incubated with 3 ml of fish extract and 1 ml of 0.04 M phosphate buffer pH 7.4 for 2 hours at 40°. Subtraction of the number of micrograms thiamin found in the digestion test from that of the control test gave the amount of thiamin destroyed.

Results. As shown by WILLIAMS et al. (1935), thiamin is split up into pyrimidinesulfonic acid and thiazole by the action of acid sulphite.

$$C_{12}H_{16}N_4OS + H_2SO_3 \longrightarrow C_6H_9N_3SO_3 + C_6H_9NOS$$

The quaternary thiazole is thereby reduced to a tertiary. The optimum of the reaction is at pH 5 and in bicarbonate buffer the reaction can be disregarded (LIPMANN, 1938). This lessens the physiological interest of the reaction. When searching other reducing substances it was found that both cysteine, glutathione and ascorbic acid in low concentrations reacted with thiamin to give a compound which could not be determined with the method of MELNICK and FIELD.

The experiments were carried out in the following way. A series of tubes, each containing 700 micrograms of thiamin, enough water and 0.04 M phosphate buffer pH 7.4 to make 4 ml, and 1 ml of the reducing solution to be tested, were incubated for 2 hours at 40°. After the incubation 5 ml of water were added to each tube, and 2 ml of this solution were used for the thiamin determination. The values obtained are directly comparable with those obtained in the analysis of fish viscera. The results of a typical series of determinations are given in Table 1.

Cysteine was about three times as active as glutathione, which was to be expected, as the cysteine equivalent of glutathione constitutes about $^1/_3$ of the molecule. Vitamin C was not quite as active. Similar series of analysis were also carried out with cocarboxylase, 960 micrograms being used in each test.[1] This amount corresponds to 700 microgram of thiamin. As demonstrated

[1] Thanks are due to the Ferrosan Corporation, Malmö, for supplies of cocarboxylase.

Table 1.

The inactivating effect of cysteine, glutathione and vitamin C on solutions of B_1.

140 microgram of B_1 give the extinction value 0.40.

Reducing substance	Extinction values after the addition of the following amounts of reducing substances in mg.				
	15	5	1	0.5	0.1
Glutathione	0	0.20	0.40	0.40	0.39
Cysteine	0	0.07	0.34	0.39	0.40
Vitamin C	0	0.30	0.40	0.41	0.40

Thiamin was allowed to react with the reducing substances for 2 hours at 40° whereupon the reaction did not proceed any longer.

by MELNICK and FIELD (1938) the phosphorylated thiamin reacted with the diazotized p-aminoacetophenon to yield coloured solutions (orange to pink). The colour was not soluble in xylol, and was read off in the water solutions using filter S 53 and 1 cm cups. The figures obtained were taken to a calibration curve constructed from the values obtained with standard solutions of cocarboxylase. The colour of the tests with cocarboxylase and glutathione was more yellow, and could only be estimated very roughly. Table 2 shows the influence of cysteine and glutathione on cocarboxylase.

Table 2.

The inactivating effect of cysteine and glutathione on solutions of cocarboxylase.

190 microgram of cocarboxylase give the extinction value 0.35.

Reducing substance	Extinction values after the addition of the following amounts of reducing substances in mg.			
	10	5	1	0.5
Glutathione	0.15	0.36	0.38	0.37
Cysteine	0.00	0.10	0.35	0.34

Cocarboxylase was allowed to react with the reducing substances for 2 hours at 40° whereupon the reaction did not proceed any longer.

On the whole cocarboxylase was not so easily reduced as thiamin. In the attempts to study the reaction, interest was focused on the results obtained by LIPMANN (1938) when he investigated the reduced thiamin. The function of the pyridine coenzymes as

hydrogen transporters in biological oxidations, as demonstrated by WARBURG and CHRISTIAN (1936), depends on the alternative hydrogenation and dehydrogenation of the double bond adjoining the quaternary nitrogen in the nicotinic acid amide part of the molecule. KUHN and VETTER (1935) showed that thiamin in the presence of Pd adsorbed 1.08 moles of H_2. LIPMANN (1938) pointed out the similarity in structure of the thiazole portion of the thiamin molecule to that extant in the pyridinium compound. He, too, was able to reduce the vitamin, either with hydrogen activated by platinum black or with hyposulphite. In phosphate solutions at pH 7.9 0.9 moles of H_2 was taken up per mole of thiamin. The reduction with hyposulphite was followed by the manometric method of HAAS (1936), in which decomposition of a bicarbonate solution by the acid formed through the oxidation of hyposulphite is measured.

$$Na_2S_2O_4 + X + 2H_2O \longrightarrow 2\,NaHSO_3 + X - H_2$$
$$NaHSO_3 + NaHCO_3 \longrightarrow Na_2SO_3 + H_2O + CO_2$$

2.7 moles of CO_2 were evolved per mole of thiamin. As only about 2 atoms of hydrogen were taken up in the presence of platinum black, the "extra acid" must result from a side reaction. WARBURG had observed a similar formation of extra acid in the hyposulphite reduction of the pyridine coenzymes, and LIPMANN followed WARBURG's explanation of that reaction in his interpretation of the reduction of thiamin. LIPMANN believed that the reduction takes place at the double bond of the quaternary nitrogen. Therefore, only 1 hydrogen atom is added. The other is split up into a proton and an electron. The latter neutralizes the positive charge of the quaternary nitrogen, and the extra proton accounts for the extra mole of acid formed.

LIPMANN and PERLMANN (1938) extended LIPMANN's earlier experiments, and demonstrated that it is the thiazole and not the pyrimidine portion of the thiamin molecule, which is reduced. LIPMANN's results were verified by STERN and MELNICK (1939), who also demonstrated that cocarboxylase was reduced by platinum black and hyposulphite in the same manner as thiamin. However, the biological significance of these results are not clear. The reduced forms of thiamin and cocarboxylase are biologically inactive and not autoxidable.

For several reasons it was of interest to determine if the reduced form of thiamin obtained by LIPMANN reacted with diazo-

tized p-aminoacetophenon. If no coloured product was formed the compound formed in the interaction between glutathione, cysteine and vitamin C on one side, and thiamin on the other could be the same as that obtained by LIPMANN. In such a case, it would also be more easily understandable why only 5—15 % of large test doses of thiamin given to animals and men, saturated with B_1, can be regained in the urine. The rest would be excreted in the form of the reduced vitamin.

The experiments were carried out in the same manner as described by HAAS. To 5 ml THUNBERG tubes were added 4 ml 1 % $NaHCO_3$ and 1 ml (700 microgram) thiamin solution. The oxygen in the solutions of the tubes was replaced by oxygen-free nitrogen by evacuation in the usual way, and then 0.2 ml of a 1.5 % $NaHCO_3$ solution containing different amounts of $Na_2S_2O_4$ was added and the tubes again filled with nitrogen. The tubes were then incubated at 30° for 2 hours. After this time the reaction should be ended (LIPMANN, 1938). The contents of the tubes were diluted to 10 ml with water and 2 ml used for analysis. The results of a typical series are given in Table 3.

Table 3.

The interaction between thiamin and hyposulphite.

2.1×10^{-3} millimole thiamin incubated for 2 hours with different amounts o hyposulphite. 140 microgram of thiamin give the extinction value 0.40.

Sample	Extinction value after incubation with hyposulphite in millimole $\times 10^{-2}$			
	0	1	2	3
$B_1 + Na_2S_2O_4$	—	0.39	0.25	0.12
B_1 in $NaHCO_3$	0.39			

When the molar relationship between thiamin and hyposulphite was 1 : 5, no inactivation of B_1 as determined with the diazo method could be observed. A similar relationship between the substance to be reduced and the reducing substance was used by HAAS in his investigation. MELNICK and FIELD used 1 mg of thiamin and 3.5 mg of $Na_2S_2O_4$. Accordingly, it would seem as if the reduced thiamin of LIPMANN could be determined with the diazo method. The inactivation of thiamin observable when greater amounts of hyposulphite are used, is probably connected with a further reaction of another type, also observed by LIPMANN and by him ascribed to the saturation of a second double bond in the thiamin molecule.

From the results of the above experiments it would not seem unprobable if the product formed in the interaction between thiamin and the reducing substances, such as cysteine and glutathione, is not the reduced form of the vitamin obtained by the addition of 1 mole of H_2 to the molecule of thiamin. It is possible that a reaction similar to that accomplished by sulphite occurs, i. e. the thiamin is split up into two halves. The same reaction occurs when the thiamin inactivating enzyme of fish viscera interacts with its substrate. Accordingly, it would not be surprising if this reaction also was a reduction. The possibility was further investigated.

First an attempt was made to inactivate the enzyme by dialysis, and to reactivate the inactive enzyme with glutathione. That the enzyme was inactivated by dialysis had previously been described by us (LIECK and ÅGREN) and by KRAMPITZ and WOLLEY. As the latter authors demonstrated, the enzyme could be partially reactivated by incubating the non-dialysable part with the dialysate. Ground, frozen viscera was extracted with an equal volume of water by shaking at 0° for 1 hour, followed by centrifugation for 20 minutes at 3,000 r. p. m. Usually 40 ml of the centrifugate were dialyzed for 24 hours against water or different buffer solutions which were renewed 3 times in all. The 5,000 ml of dialysate were concentrated in vacuum at 15° to 40 ml. In the enzymatical tests the neutralized non-dialysable solution was diluted with water, an equal volume of concentrated dialysate or a solution of glutathione, and incubated with vitamin in the usual way. Table 4 gives some of the results.

The non-dialysable portion of the fish extract after the dialysis against water or acetate buffer pH 5.5 was often fairly active At the same time it was observed that a precipitation was formed during the dialysis The precipitate was solved at the neutralization of the non-dialysable portion to pH 7.4. If the precipitate was centrifuged before neutralization, the non-dialysable extract was inactive. Obviously the enzyme was precipitated in the vicinity of pH 5, either depending on adsorption to other isoelectrically precipitated proteins or on the insolubility of the enzyme at its own isoelectric point. The latter assumption seemed to be the more probable one, as demonstrated in experiments with the partially purified enzyme to be described later on. When the dialysis was carried out with more acid extracts, a better separation of enzyme and coenzyme was obtained. From the analysis of Table 4 it is also evident that the active dialysable portion can not activate an extract from a fish which, according to the che-

Table 4.

The thiamin destroying potency of dialyzed fish extracts.

Fraction of dialysate	Dialyzed against	Amount[1] used ml	Thiamin destroyed[2] γ	Fish
Extract		3	> 440	Carp
Non-dialysable portion	Water	1.5 + 1.5 aq	175	»
Non-dialysable + dialysable portion	»	1.5 + 1.5	210	»
Extract		3	525	Ide + rudd
Non-dialysable portion	Water	1.5 + 1.5 aq	70	»
Non-dialysable + dialysable portion	»	1.5 + 1.5	240	»
Extract of melt + dialysable portion of ide and rudd		1.5 + 1.5	0	
Non-dialysable portion	Acetate pH 5.5 0.01 N	1.5 + 1.5 aq	310	»
Non-dialysable + dialysable portion	» »	1.5 + 1.5	350	»
Non-dialysable portion	» pH 4.4	1.5 + 1.5 aq	90	»
Non-dialysable + dialysable portion	» »	1.5 + 1.5	260	»
Extract		3	> 530	Rudd
Non-dialysable portion	Citrate pH 2.0 0.01 N	1.5 + 1.5 aq	44	»
Non-dialysable + dialysable portion	» »	1.5 + 1.5	400	»
Non-dialysable portion	» pH 3.5	1.5 + 1.5 aq	175	»
Non-dialysable + dialysable portion	» »	1.5 + 1.5	350	»

[1] The volume of the dialysate was adjusted to the same as the non-dialysable portion.

[2] Each reaction mixture contained originally 700 γ thiamin.

mical analysis of LIECK and ÅGREN, does not contain the enzyme or possibly only the apoenzyme. In several experiments partially inactivated non-dialysable portions were incubated with glutathione in order to see whether the enzyme could be reactivated with glutathione to the same extent as with the dialysate In Table 5 some of the results are given.

Obviously, glutathione restores some of the original activity of the enzyme. It is possible that glutathione reactivates the enzyme through a reduction and that the enzyme only in this state can be active. In control experiments with extracts from fishes which did not contain the enzyme, it was observed that the addition of 1—2 mg of glutathione per 1.5 ml of extract was not followed by any inactivation, when the solution was incubated with thiamin. The fish extracts were diluted to contain the same

Table 5.

The activating effect of glutathione on solutions of enzyme partially inactivated by dialysis.

Fraction of dialysate	Dialyzed against	Amounts[1] used ml	Thiamin destroyed[2] γ	Fish
Non-dialysable portion....	0.01 N acetate pH 4.4	1.5+1.5 aq	0	Rudd+crucian
Non-dialysable + dialysable portions.....	»	1.5+1.5	175	» »
Non-dialysable portion + 2 mg glutathione.....	»	1.5+1.5	175	» »
Non-dialysable portion....	»	1.5+1.5 aq	105	Carp + rudd
Non-dialysable + dialysable portions.....	»	1.5+1.5	175	» »
Non-dialysable portion + 1 mg glutathione.....	»	1.5+1.5	260	» »

[1] The volume of the dialysate was adjusted to the same as the non-dialysable portion.

[2] Each reaction mixture contained originally 700 γ thiamin.

amount of protein nitrogen as the non-dialysable portions of active fish in Table 5. The assumption that the fish enzyme functions by a reductive splitting up of the thiamin molecule would certainly be strengthened if the glutathione effect could be demonstrated on enzyme preparations which had been inactivated in a different way. It was decided to investigate if the enzyme could be inactivated by hydrogen peroxide, and afterwards reactivated by glutathione. As the fish extract always decomposed the added hydrogen peroxide, it was necessary to purify the enzyme in some way Isoelectric precipitation was first tried.

Frozen, ground viscera was extracted with an equal volume of water by shaking at 0° for 1 hour, followed by centrifugation for 30 minutes at 3,000 r. p. m. This extract was acidified by stage, and the precipitates centrifuged for 20 minutes at about 15,000 r. p. m. The precipitates were solved in water and neutralized to pH 7.4. 3 ml samples were taken for analysis in the usual way. The results of a typical fractionation are given in Table 6.

The enzyme activity was partially adsorbed on several protein fractions precipitated from the solution, but the main fraction of activity always precipitated at pH between 5.0—5.3. By this procedure the enzyme activity calculated per mg of nitrogen was purified about ten times The solution of the isoelectrically pre-

[1] For a gift of crystalline catalase I am greatly indebted to dr K. Agner of the Department of Medical Chemistry at Karolinska institutet, Stockholm.

Table 6.

Isoelectric precipitation of the thiamin inactivating enzyme.

Fraction of enzyme solution	Solved in ml	Thiamin destroyed per 3 ml solution[1] γ
Extract	40	> 550
Precipitate at pH 5.8	20	190
Precipitate at pH 5.3	20	210
Precipitate at pH 5.0	20	> 520
Precipitate at pH 4.7	20	140
Centrifugate from 4.7	30	350

[1] Each reaction mixture contained originally 700 γ thiamin.

cipitated enzyme activity still contained small amounts of an enzyme which decomposed hydrogen peroxide. The enzyme was further purified by fractionated precipitation with ammonium sulphate of a centrifugate from isoelectric precipitation at pH 5.6. As the catalase activity precipitates by saturation to 0.6 with ammonium sulphate, the thiamin destroying activity precipitating between 0.6—1.0 saturation with ammonium sulphate was collected. The inactivation experiments were carried out in the following way. To 6 ml of the cooled enzyme solution in THUNBERG tubes were added 0.4 ml of 5 and 10 per cent. solutions of hydrogen peroxide. After 10 minutes 0.4 ml was again added and after a further 10 minutes the hydrogen peroxide was decomposed by the addition of crystalline catalase. The tubes were shaken for 15 minutes at 0°. The enzyme activity of some tubes was directly analyzed and the other were incubated with 0.2 ml of a neutralized solution of glutathione for 30 minutes at 0°. As usual 3 ml of the enzyme solution + 1 ml of thiamin solution + 1 ml of 0.04 M phosphate buffer were incubated for 2 hours at 40° and then precipitated with 5 ml of 20 % trichloracetic acid. The controls were precipitated without incubation. Results from series of analysis are given in Table 7.

Glutathione restored the activity of the enzyme. In control experiments with extracts of fishes which did not contain the enzyme according to chemical analysis of LIECK and ÅGREN, the viscera extracts were precipitated isoelectrically as described above. The fractions precipitated at pH 5.3—5.0 were solved to contain the same amount of protein nitrogen as the active solutions analyzed in Table 7. After the addition of 5—10 mg of

Table 7.

Inactivation and reactivation of the enzyme by hydrogen peroxide and glutathione.

Enzyme solution treated with	Thiamin destroyed[1] γ	Nitrogen content mg	Remark
Untreated solution.....	520	0.92	Enzyme solution purified by isoelectric precipitation
10 % H_2O_2.....	320	»	»
10 % H_2O_2 + glutathione, 10 mg.....	470	»	»
Untreated solution.....	530	0.23	Enzyme solution purified by fractionated precipitation with ammonium sulphate
5 % H_2O_2.....	175	»	
5 % H_2O_2 + 5 mg glutathione.....	560	»	»

[1] Each reaction mixture contained originally 700 γ thiamin.

glutathione to the solutions, treated with 5 and 10 % H_2O_2 and catalase, followed by incubation for 2 hours with 700 microgram of thiamin, no inactivation of the vitamin was observed.

Discussion.

The physiological significance of the effect exhibited by glutathione, cysteine and vitamin C on thiamin may be somewhat discussed. From the results obtained in the present investigation it is obvious that both free and phosphorylated thiamin is inactivated when incubated with five to ten times greater concentrations of the reducing substances. The thiamin concentration in blood is determined by

Sinclair (1939) to 1 microgram thiamin and 7 microgram cocarboxylase (100 ml blood)

Euler et al. (1940) 1—2 microgram thiamin and 7—15 microgram codarboxylase (100 ml blood)

Widenbauer (1939) 10 microgram thiamin and 2—5 microgram cocarboxylase (100 ml serum)

Bang (1944) 5—14 microgram thiamin including cocarboxylase (100 ml blood)

SINCLAIR also demonstrated that most of the cocarboxylase of the blood is proteinbound. In 1942 both CAJORI and KENSLER et. al. demonstrated that carboxylase is not inhibited by cysteine. On the contrary, the enzyme is activated. From this brief survey it is obvious that the inactivating effect of glutathione and cysteine will probably be accomplished by interaction mainly with the free thiamin. The glutathione concentration in blood has recently been estimated by BICHEL (1944) to about 25—50 mg per 100 ml of blood. The concentration of glutathione is at least about 1000 times greater than that of thiamin and obviously large enough to inactivate free thiamin. This may be one of the reasons why only 5—15 % of the thiamin given to individuals "saturated" with thiamin can be regained in the urine, when the usual chemical methods for thiamin determinations are used. The course of injected vitamin B_1 in the body has also been studied by using synthetic thiamin made from radioactive methods (BORSOOK et al., 1940). After injecting 16 mg of radioactive B_1 into a normal subject it was found that after 6 days 61 % of the radioactive sulphur could be detected in the urine and 11 % in the faeces; 28 % could not be accounted for. The difference between the 61 % in this experiments and the 10 % usually found after per oral administration of similar amounts of B_1 may at least partly correspond to thiamin destroyed by reducing substances such as glutathione, cysteine and vitamin C.

No attempt has as yet been made to determine the nature of the compound formed in the reductive inactivation of thiamin by glutathione and cysteine. It is probably not the compound described by LIPMANN, since that substance still seems to give the colour reaction of McCollum-Preblubda. The process will be further studied. In the meantime it may be suggestive that the enzymatical inactivation of thiamin also seems to be a reductive reaction as described above. In this process the vitamin is split up into the two halves and it is not impossible that glutathione and cysteine behave in the same way. The function of the enzyme in the metabolic processes of the body is not clear at all, but a suggestion may be made. It is reported by MINZ (1938) that electric stimulation of isolated nerves releases thiamin. The results were verified and extended in a series of paper of v. MURALT and coworkers (1942). No definite explanation of the function of thiamin in this process has been given. BRIEM (1939) found that thiamin increases the action of acetylcholine. This may be

explained by the observation of GLICK and ANTOPOL (1939) that cholinesterase was inhibited by thiamin, thereby slowing down the hydrolysis. On the other hand KUHN et al. (1939) reported that acetylated thiamin acted on intestinal stripes in a way similar to acetylcholine. If thiamin is engaged in the transmission of electric stimulations in nerves it may be necessary to abolish the effect in the same manner as acetylcholine is hydrolyzed by cholinesterase. This presumes that the thiamin-inactivating enzyme is videly distributed, a circumstance which cannot be investigated with a semi-micro method such as that of MELNICK and FIELD. On that account the distribution of the enzyme has also been investigated with the Phycomyces test. The results will be published elsewhere.

Summary.

Thiamin and free cocarboxylase interact with reducing substances such as glutathione, cysteine and vitamin C. In the reaction the vitamin B_1 is changed into a compound or compounds which cannot be determined with the colorimetric method of MELNICK and FIELD. The compound is probably not identical with LIPMANN's reduced vitamin. The activity of the fish enzyme which destroys thiamin is partly inactivated by oxidation with hydrogen peroxide in the cold, or with dialysis, and the activity can be restored subsequently by the addition of glutathione.

The writer acknowledges the valuable assistance of Mr. EKLUND throughout the investigation.

References.

BANG, H. O., B_1-vitaminstudier. Köpenhamn 1944.
BICHEL, J., Acta Med. Scand. 1944. *117*. 474.
BORSOOK, H., E. R. BUCHMANN, J. B. HATSCHER, D. M. YOST, and E. M. MILLAN, Proc. Nat. Acad. Sci. Wash. 1940. *26*. 412.
BRIEM, H. J., Pflüg. Arch. ges. Physiol. 1939. *242*. 450.
CAJORI, F. A., J. Biol. Chem. 1942. *143*. 357.
EAKIN, R. E., E. E. SNELL, and R. J. WILLIAMS, Ibidem 1941. *140*. 533.
v. EULER, H., F. SCHLENK, and B. HÖGBERG, Uppsala läkaref. förh. 1939/40. *45*, 301.
HAAS, E., Biochem. Z. 1936. *285*. 368.

KENSLER, C. J., N. F. YOUNG, and C. P. RHOADS, J. Biol. Chem. 1942. *143*. 465.
KUHN, R., and H. WETTER, Ber. dtsch. Chem. Ges. 1935. *68*. 2375.
KRAMPITZ, L. O., and D. W. WOLLEY, J. Biol. Chem. 1944. *153*. 11.
KUHN, R., T. WIELANDT, and H. HUENSCHMAUN, Hoppe-Seyl. Z. 1939. *259*. 48.
LIECK, H., and G. ÅGREN, This Journal. *1944*. *8*. 203.
LIPMANN, F., Nature. 1936. *138*. 1097.
LIPMANN, F., and G. PERLMANN, Amer. J. Chem. Soc. 1938. *60*. 2574.
MELNICK, D., and H. FIELD, J. Biol. Chem. 1938. *127*. 515.
MINZ, B., C. R. Soc. biol., Paris 1938. *127*. 1251.
v. MURALT, A., Pflüg. Arch. ges. Physiol. 1942. *245*. 604.
ROSE, W. C., Physiol. Rev. 1938. *18*. 109.
SEALOCK, R. R., A. H. LIVERMORE, and C. A. EVANS, J. Amer. Chem. Soc. 1943. *65*. 935.
STERN, K. G., and J. L. MELNICK, J. Biol. Chem. 1939. *135*. 365.
WARBURG, O., and W. CHRISTIAN, Biochem. Z. 1936. *287*. 291.
WIDENBAUER, F., Klin. Wschr. 1939. *18*. 1392.
WILLIAMS, R. E., J. C. WATERMAN, J. C. KERESZTESY, and E. R. BUCHMAN, J. Amer. Chem. Soc. 1935. *57*. 536.
ÅGREN, G., This journal. 1944. *8*. 305.

From the Kristineberg Zoological Station, Sweden.

Rate of Renewal of the Fish Skeleton.

By

G. HEVESY.

Received 30 October 1944.

The phosphorus atoms present in the organism migrate from molecule to molecule and from organ to organ. The rate of migration greatly depends upon the nature of the molecules involved and on the organ in which they are located, the phosphorus atoms finding their most lasting abode in the skeleton. The mineral constituents of the skeleton are located in apatite-like crystallites which have a size of about 10^{-6} cm. From the phosphate ions present in these crystallites, only those located in the uppermost molecular layer can come into direct contact with the lymph or the plasma and, thus, participate in an interchange with the phosphate present in the plasma (lymph). The residual part of the crystallites will be prevented from participating in such an interchange by the fact that, at body temperature, the rate of penetration of ions into an apatite-like crystal is entirely negligible. The replacement of the bulk of the phosphate or other ions present in the apatite-like crystals can only take place by a partial or total dissolution of the crystallite followed by a crystallization process leading to a partial or total formation of new crystallites. As was to be expected and also was shown when making use of isotopic indicators, such a "biological recrystallization" of the bone apatite is a very slow process. This process is made possible by the fact that the concentrations of phosphate and of other constituents of the plasma vary. Intake of food increases the phosphate content of the plasma and the lymph, and so do numerous biochemical processes leading to an enzymic splitting of organic phosphorus compounds. The plasma phosphate, for example, increases after

intense muscular action, though the low phosphate permeability of the muscle cells (HEVESY and REBBE 1940) much reduces the exodus of the phosphate ions split off during muscular action. On the other hand, excretion of phosphate acts in the opposite direction, and so do all those numerous biochemical processes in the course of which phosphate becomes incorporated with organic compounds, from which processes the decrease of the phosphate content of the plasma under the action of insulin is possibly the most conspicuous one (cf. KJERULF-JENSEN and LUNDSGAARD 1943).

Not only do the phosphate and the equally important calcium concentrations of the plasma fluctuate, but the same applies to the concentration of phosphatase and other enzymes regulating the phosphorus metabolism. Such enzymes act on the bone formation not only by regulating the phosphate concentration of the plasma, but possibly also in a more direct way, as suggested by ROBISON's (1912) early studies on bone formation. More recent work by ROCHE and MOURGUE (1939) leads to the result that a fracture of the rat's femur involves a loss of appreciable amounts of the mineral constituents of the femur followed by an opposite process after the lapse of about one month. In the first weeks, phosphatase activity of the bone is also enhanced. ROCHE and MOURGUE made the very interesting observation that the fracture of the left femur leads not only to an initial decrease in the mineral content of the fractured left bone, followed later by a reversal of this process, but a similar behaviour is also shown by the intact right femur. The enhanced phosphatase activity of the bone tissue may be due to an increased magnesium concentration produced by osteolysis following the fracture. Thus, even a fluctuation in the magnesium content of the plasma may promote the rate of renewal of the skeleton.

Fluctuations in the phosphate, calcium, magnesium, and phosphatase contents of the plasma thus make possible a biological recrystallization of bone apatite and, correspondingly, a renewal of the skeleton. That this process, which can be followed by making use of isotopic indicators, was found to be a slow one is easy to understand. The bone apatite contains a very appreciable part of the body's calcium and phosphorus contents, and these constituents are present in a crystalline state. Dissolution and formation of such crystallites may be expected to be a slow process. Furthermore, we must envisage

the probability that a partial dissolution of a crystallite may be followed by a new formation of some molecular layers which protect the underlying part of the crystal from further changes. This process can often be repeated and leads to a repeated renewal of a fraction of the crystallites, while the remaining part of the crystal remains unchanged. The pronounced difference in the rate of renewal of epiphysial and diaphysial tissues found by various workers is due mainly to the better circulation taking place in the soft epiphysial bones but, possibly, to some extent to the smaller size of the crystals of the epiphysial tissue which favours an interchange between lymph (plasma) and bone phosphate.

In this paper are communicated the results of experiments carried out with the aim of measuring the rate of renewal of the mineral constituents of the fish skeleton. However, a short survey of the results hitherto obtained for the rate of renewal of the skeleton of mammalia will first be given.

Rate of Renewal of the Skeleton of Mammalia.

As a result of the administration of labelled phosphate (phosphate containing a minute percentage of the radioactive phosphorus isotope ^{32}P), the "free" phosphate of the blood plasma soon becomes labelled, and the same applies to the extracellular fluid of the organism in view of the swift passage of phosphate ions through the wall's capillaries. As the plasma and the lymph contain labelled phosphate, all bone apatite formed after the administration of labelled phosphate is bound to be labelled. In the extreme case, when all mineral bone tissue is formed after the administration of labelled phosphate, 1 mg bone P will have he same ^{32}P content, and thus the same radioactivity, as 1 mg plasma P. Thus, the ratio of the specific activity (activity of 1 mg) of the skeleton P and the specific activity of the plasma P is a measure of the rate of renewal of the skeleton.

When determining the rate of renewal we must take due regard to the fact that the specific activity of the plasma phosphorus does not remain constant, but decreases strongly in the course of the experiment, owing to successive interchanges of the plasma phosphorus with the phosphorus atoms of the various compounds present in the organs and also to excretion of phosphate. As the rate of interchange is different for different compounds

and also for different organs, the calculation of the decrease of the specific activity of the plasma P with time encounters very great difficulties. The most direct way to eliminate the above mentioned difficulty is to administer repeatedly an appropriate amount of labelled phosphate, and, with the aid of this procedure, to keep the plasma phosphate at a constant level throughout the experiment. The results of such experiments (HEVESY et alia 1940) carried out on rabbits are seen in Table 1.

Table 1.

Extent of renewal of the skeleton of a rabbit in the course of 50 days.

Fraction	Per cent renewed
Femur epiphysis inorganic P	29.7
Femur diaphysis inorganic P	6.7
Tibia epiphysis inorganic P	28.6
Tibia diaphysis inorganic P	7.6
Costa inorganic P	27.5
Scapula inorganic P	43.8
— — — — — — — — — — — —	—
Femur epiphysis phosphatide P....	100

As seen from the figures, the degree of renewal of the mineral constituents of the skeleton in the course of 50 days amounts to 29 per cent in the case of the epiphysis of the tibia and to as little as 8 per cent for the diaphysis, while phosphatides extracted from the bone tissue are entirely, and possibly even several times, renewed in the course of the experiment.

The experiments mentioned above were carried out with fully grown rabbits, as in a growing organism the presence of labelled atoms cannot be interpreted exclusively as the result of a renewal process. It will also be due to the formation of *additional* tissue during the experiment. All molecules formed in a growing, labelled organism are, indeed, bound to become labelled. It is of importance, therefore, to carry out experiments on the reneval of the skeleton on adult animals.

The incorporation of labelled phosphate into mineral components of the bone is a very intricate process. Between the uppermost molecular layer of the apatite crystallites in contact with plasma or lymph, an exchange equilibrium can be established almost immediately. This means that the specific activity of the P present in these layers will promptly follow all changes

in the specific activity of the plasma phosphorus. In most experiments with labelled phosphate, the active preparation is administered at the start of the experiment. After subcutaneous injection or administration by mouth, an increase in the plasma activity will take place in the initial phases of the experiment and a decrease throughout the later phases. Thus, the activity of the uppermost layer of the bone apatite is strongest in the early phases of the experiment in which the plasma is strongly active. Crystallites, however, formed in a later stage of the experiment from an active plasma, will contain comparatively large amounts of ^{32}P in view of the high phosphorus content of the total crystallites compared with the phosphorus content of the uppermost molecular layer. In view of the stability of the crystallites, their ^{32}P content will be well conserved and will not follow or follow only slowly the changes in the activity of the plasma phosphorus.

Beside formation of entire crystallites from the labelled plasma we have also to consider the case of partial formation of crystallites. Some molecular layers are dissolved and replaced by layers formed by crystallization from labelled plasma. The newly formed layers will be active, but not the layers lying below. These layers will be protected from all action of the labelled plasma and, thus, from renewal. They will form a stable core for the crystallites and so will also all crystallites that are not in contact with plasma or lymph.

MANLY and his colleagues (1940), who carried out extensive studies into the uptake of ^{32}P by the mineral constituents of the bone where the activity was administered at the start of the experiment, estimate the share of labile and stable fractions of the bone tissue by comparing the activity of the blood (not of plasma) P and of the bone mineral of rats. They estimate $^1/_5$ of the ^{32}P content to be present after the lapse of 20 days in the labile portions of the epiphysis, the rest being found in the stable portion. The estimation of such magnitudes encounters great difficulties in view of the very complicated way in which the labelling of the bone tissue takes place. The degree of renewal of the mineral constituents of the different parts of the skeleton which takes place within a time interval can, however, be determined in the way described on p. 237. The degree of renewal in these experiments means the percentage of bone tissue newly formed once or several times since the start of the experiment.

Rate of Renewal of the Fish Skeleton.

We investigated the rate of renewal of the skeleton of sticklebacks (*Gasterosteus aculeatus*). These fish, weighing 1—3 g, have a lifetime of about one year and can be expected not to grow any longer when one year old. Their small size has the advantage that the use of a large sea-water volume and, thus, an unduly strong solution of labelled phosphate, can be avoided. Our experiments, in which the sticklebacks were kept for up to six weeks in labelled sea-water, were carried out with radio-phosphorus having an initial activity of 0.05 milli-Curie, which was found to be ample to give an active skeleton.

The small size of the fish facilitates, furthermore, their ashing, which is to be carried out when we want to determine the total phosphorus content or the total ^{32}P content of the fish. Wet ashing was carried out by heating with 1 ml of conc. sulphuric acid containing some nitric acid and, in the last phase of the experiment, some hydrogen peroxide. 24 sticklebacks were kept in 3 litres of sea-water to which 10 ml of a radioactive solution containing 0.05 milliCurie and 3.4 mg of sodium phosphate (pH = 7.6) were added. After the lapse of 6 weeks, this activity declined to $^1/_8$ of its initial value. The water was daily renewed, as was its labelled phosphate content.

The sticklebacks were investigated at different intervals. After killing the fish and washing it very carefully with sea-water, the liver was taken, and the "free" phosphate was extracted from it with cold 5 per cent trichloroacetic acid. The solution was then brought up to 25 ml. While the "free" phosphate content of 20 ml was precipitated as magnesium ammonium phosphate, the activity of which was measured, the residual part used in a colorimetric determination of the free P content of the extract. The activity measurements are much simplified when the samples have about the same weight. To obtain such samples, we added to the above mentioned 20 ml so much sodium phosphate that the precipitate obtained weighed 60 mg. As mentioned above, the determination of the rate of renewal is based upon the comparison of the activity of 1 mg of free plasma P and 1 mg of mineral skeleton P. It is, however, extremely difficult to secure blood from a fish weighing a couple of grams and, therefore, we replaced the determination of the activity of the plasma P by a determination of the activity of the free liver P. In view of the great ease with which phosphate ions penetrate the liver cells and vice versa, the activity level of the free phosphate P of the liver hardly differs from the activity level of the free phosphate of the plasma. The writer is much indebted to Mr. TRYGGVE GUSTAVSON for his very effective help in removing the livers, weighing 45—70 mg.

To remove the organic constituents of the skeleton, we treated the bones with boiling glycol containing 6 g KOH per 100 ml for several hours, until the bones showed the total absence of non-mineral constituents. The bones were dried at 105° and dissolved in 2 ml 0.5 N HCl. The solution was brought to 25 ml and an aliquot was used, as described above, in the radioactive measurements, while the other

aliquot was taken for colorimetric determination. The writer is much indebted to Miss KERSTIN ÖHLIN and Miss MARIANNE ANDERSSON for their very effective assistance in these determinations.

Uptake of Labelled Phosphate by the Fish.

The uptake of labelled phosphate by the fish most probably takes place either through the gills or through the digestive tract. While water passes the surface of the gills, some phosphate may reach the circulation. An increase of the water volume passing the gills may in this case be expected to lead to an increase in the phosphate uptake. Therefore, we have compared the phosphate uptake by fish kept in water rich in oxygen with the uptake of phosphate by fish in water containing but very small amounts of oxygen. While a group of fish was kept for a day in 2 litres of labelled seawater saturated throughout the experiment, another group was kept in 2 litres of labelled sea-water to which no oxygen was added and in which other sticklebacks were previously kept in order to remove much of its air content. As seen in Table 2, the average uptake of ^{32}P by the two groups of fish practically does not differ.

Table 2.

Uptake of ^{32}P by fish kept in oxygen-rich and oxygen-poor water in the course of 24 hours.

Weight of fish in g	Total activity present in 1 g fish	
	water rich in oxygen	water poor in oxygen
0.82	20	
2.07	15	
1.77	25	
1.53	21	
1.25	17	
1.03	24	
1.15	16	
1.30	21	
0.91		23
0.88		21
1.28		13
2.63		19
1.71		26
1.51		22
1.24		20
1.56		24
Mean value	20	22

The fact that no significant difference was found in the uptakes of ^{32}P from waters rich and poor in oxygen does not prove conclusively that the main uptake of ^{32}P does not take place through the gills, as it is possible that the organism reacts to oxygen shortage in the water not by an increase in the water circulation through the gills, but by an enhanced oxygen extraction from the water. However, the above result induces us to draw our attention to the other probable way of entrance, the digestive tract, which, as marine fish drink large quantities of the water in which they swim, is the most probable path of ionic uptake by such fish.

HOMER SMITH (1930, 1931) added phenol red to aquarium water and found that the dye became concentrated in the intestine and also that it could not be absorbed through the gills and the skin. By measuring the concentration of the dye he was able to calculate the extent of water absorption taking place.[1] An eel weighing 143.5 g, was found to have swallowed in the course of 20 hours 12.3 ml of sea-water. A number of experiments on eels and sculpins show that per kg. of weight those fish swallow from 40 to 225 ml of sea-water per day. The minimum swallowing observed is thus 0.04 ml per g per day. The amount of labelled phosphorus present in 0.04 ml of water is that found by us in a stickleback weighing 1 g after the lapse of 16 days. If the amount of water swallowed by the stickleback is not still smaller than the smallest amount observed in eels and sculpins, then we have to conclude that only a minor percentage of phosphate present in the swallowed water is absorbed by the stickleback. This result is by no means improbable. Though water is very easily absorbed, HOMER SMITH found that only 81 per cent of the water swallowed by the eel had been absorbed and, furthermore, that, while monovalent ions such as Na^+, K^+, and Cl^- were absorbed to a very large extent, divalent ions such as Ca^{++}, Mg^{++}, and $SO_4^=$ were not, these ions being concentrated in the rectal fluid. In relation to chlorine, sulphate was, for example, concentrated 24 times in the rectal fluid. (Cf. A. KROGH, 1939). The result obtained by us indicates a similar fate of the phosphate ions.

A fish weighing 0.92 g took up in the course of 8 days $4 \cdot 10^{-3}$ per cent of the activity of the labelled sea-water and, thus, as

[1] A detailed account of the work of HOMER SMITH is given by KROGH (1939).

the phosphorus content of the sea-water was 880 γ (730 added + 150 present beforehand), 3.5 γ of phosphorus were taken up mainly as sodium phosphate by the fish. The amount of phosphate taken up by the fish during the same time varies appreciably from fish to fish. However, these variations do not influence the results obtained for the rate of renewal since, when calculating this magnitude, the skeleton activity and the liver activity of the same fish are considered.

Gasterosteus aculeatus is a fish provided with a skin armour.[1] In several cases, we investigated the armour, the cranium, and the vertebra separately.

Extent of Renewal of the Skeleton.

The method of calculating the extent of renewal of the skeleton is shown by the following example.

Duration of the experiment = 16 days. Average temperature = = 16.6°. Fresh weight of the fish = 1.49 g. Weight of the fresh liver = 50 mg.

Free P content of the liver = 21 γ.

Total P content of the skeleton = 3.98 mg.

Activity of the skeleton P = 29.2 counts per minute.

Activity of the free liver P = 8.8 counts per minute.

$$\frac{\text{Activity of 1 } \gamma \text{ skeleton P}}{\text{Activity of 1 } \gamma \text{ free liver P}} = \frac{0.00733}{0.418}.$$

Activity of 1 γ skeleton P in percentage of the activity of 1 γ liver P 1.8.

The figure obtained is not strictly identical with the percentage of the skeleton which, in the course of the experiment, is renewed, as we compared the specific activity of the bone P at the end of the experiment with the specific activity of the free liver P at the same date, while we should have considered the average value of the specific activity of the free liver P prevailing throughout the experiment. This magnitude is not known, but cannot be less than $^1/_2$ of the *final* value. Therefore, we have to multiply, the result of 1.8 per cent arrived at by a figure which is less and probably appreciably less, than 2 in order to arrive at a correct renewal percentage which, thus, amounts to 3—4 per cent in the course of 16 days. This percentage of the skeleton

[1] A detailed description of the armour is given by F. Roth (*1920*).

was renewed once or several times, while the remaining 96—97 per cent of the skeleton remained unchanged.

The fish was kept in 3 litres of sea-water to which 3.4 mg of labelled phosphate, corresponding to 0.73 mg P and having an activity of $1.6 \cdot 10^6$ counts, were added (measured the same day as the activity of the liver and that of the skeleton). Water and activity were daily renewed.

1 γ free liver P was found to contain $\frac{1}{4 \cdot 10^6}$ part of the activity and, thus, from the free P extracted from the liver $2 \cdot 10^{-4}$ γ were such which originate from the labelled phosphate added to the sea-water. The total free liver P contained $\frac{1}{1.8 \cdot 10^5}$ part of the activity added to the water. The skeleton contained $1/5.3 \cdot 10^4$ part of the activity added to the water, thus of the 3.98 mg. P present in the skeleton, $1.4 \cdot 10^{-2}$ γ were such which originate from the labelled phosphate added to the sea-water.

The results obtained for different parts of the skeleton and various fish are computed in Table 3.

Table 3.

Percentage ratio of the activity of 1 mg skeleton P and 1 mg free liver P.

No. of experiment	Fresh weight of the fish in g	Time in days	Part of the skeleton	Percentage ratio of the activity of 1 mg skeleton P and 1 mg liver P
19	1.83	17	Skull Vertebrae Armour	1.7 1.1 1.2
18	2.20	20	Armour	2.3
21	1.78	20	Skull Armour	2.7 2.1
22	1.21	21	Skull Vertebrae Armour	2.4 3.4 2.6
23	1.33	30	Vertebrae Armour	4.1 2.4
24	1.41	31	Armour	4.2

From the above figures, the average value for the percentage ratio of the activity of 1 mg skeleton P and 1 mg liver P works out to be 2.5 per cent in the course of 22 days. To obtain the

percentage renewal of the fish skeleton in the course of 22 days, we have to multiply the above figure (cf. p. 242) by a figure which is less than 2. The rate of renewal thus lies between 2.5 and 5 per cent.

Uptake of Labelled P by the Eggs.

In several fish, a large number of eggs was found and in many cases the weight of the eggs constituted a very appreciable percentage of the weight of the fish. In experiment No. 16 B, the eggs' weight was found to be 0.92 g out of a weight of 2.44 g of the fish (including eggs), i. e. 38 per cent. The percentage ratio of the activity of 1 mg average fish P (minus eggs) and 1 mg free liver P was found to be 6.3. For the percentage ratio of the activity of 1 mg egg P and 1 mg liver P, 90 was obtained. Thus, almost all P of the atoms present in the egg was incorporated in the eggs in the course of the last 23 days. The total P content of the fish without its eggs was found to be 17.78 mg, or 1.17 per cent, while the corresponding figures for the eggs were 2.7 and 0.29.

Fish No. 16 had a weight of 1.07 g including its eggs, which made up 40 per cent of the total weight. Liver and heart were strongly degenerated, the liver weighing only a few milligrams. The duration of the experiment was 16 days, the activity of the eggs amounting to 12.8 per cent of the total activity. In the above case (No. 16 B), the corresponding ratio was 18.3. Thus, after the lapse of 16 days, only about $^2/_3$ of the egg P was found to be incorporated in the eggs in the course of the experiment, while in experiment No. 16 B., after the lapse of 23 days, almost the whole P content of the eggs was deposited (through growth or renewal) in the course of the experiment.

In experiment No. 16 A., the percentage of P taken up in the course of the experiment from the water was found to be $1/3.9 \cdot 10^4$ of the amount present, corresponding to $2.2 \cdot 10^{-2}$ γ.

In experiment No. 16 B, $1/2.2 \cdot 10^4$ of the water P was taken up by the fish, amounting to $4.4 \cdot 10^{-2}$ γ.

Discussion.

The average value for the degree of renewal of the fish skeleton in the course of 22 days was found to be 2.5—5 per cent, the lower value being the more probable. This means that, while

2.5—5 per cent of the skeleton were renewed once or several times — a part of this percentage was certainly renewed very frequently — at least 95 per cent of the skeleton remained entirely unchanged. When arriving at this conclusion, we assumed that no additional growth of the skeleton took place in the course of the experiment. As such additional growth would take place from a labelled plasma, all newly formed skeleton might be expected to be labelled, and what we interpreted as a renewal of the skeleton might in such a case be due to additional bone formation in the course of the experiment. We could not find any evidence for a growth of the skeleton or a growth of the fish taking place in the course of the experiment. It is very difficult, however, to exclude the possibility of an increase in the mineral constituents of the skeleton by a few percent. The above mentioned 95 per cent therefore have to be considered a lower limit for the part of the skeleton remaining unchanged after three weeks.

In the case of the fully grown rabbit, about 10 per cent of the skeleton were found to be renewed in the course of only 9 days (HEVESY et alia 1940). The great difference in the renewal rate of the skeleton of the rabbit and that of the fish is presumably due to the great difference in the body temperature. The renewal of the skeleton is partly a "physical" replacement process between the phosphate of the uppermost molecular layer of the bone crystallites and the phosphate of the plasma, and partly the effect of a "biological recrystallization". Crystallites or parts of crystallites go into solution and new crystallites are wholly or partly formed by crystallization from the plasma. In experiments of long duration, the interchange mostly takes place by biological recrystallization. Now, such a process may be expected to be strongly influenced by the body temperature and to take place at a higher rate at 37° than at 16°.

It is interesting to note that even in experiments of only a few hours' duration, increase of temperature was found to promote the radio-phosphorus uptake by the bones. The tibia of the frog (HEVESY et alia 1940) was found to take up nearly 1½ times as much radio-phosphorus at 22° as at 0°.

The application of the method of radioactive indicators necessitates the uptake of measurable amounts of the indicator by the organism. If only this condition is fulfilled, the amount of the indicator taken up by the organism does not influence the results obtained by the indicator method, results which are,

in the case discussed here, based on the comparison of the specific activity of the bone P and that of the free plasma (liver) P. Rate of uptake and renewal rate present entirely different problems.

We found that a fish weighing about 1 g took up, in the course of 16 days, $1/4 \cdot 10^4$ part of the phosphorus added to the water i. e. $2 \cdot 10^{-5}$ mg. As the water volume was 3,000 ml., the amount of P taken up by the fish from water is equivalent to the amount of P present in 1/13 ml water. The amount of water taken up by the fish in the course of 16 days is presumably much larger than 1/13 of its body weight (cf. p. 241) and, thus, only a minor part of the P content of the water swallowed is absorbed.

The P content of the eggs was found to be composed mostly of P atoms incorporated in the course of the experiment. In the case of the hen's egg (HEVESY and HAHN 1938), most of the P atoms were found to be noninterchangeable and, correspondingly, the presence of a large percentage of labelled P in the hen's egg proves the formation of a large part of the egg from labelled plasma. Should this conclusion be applicable to the fish egg, which is problematic however, we might conclude that most of the fish egg material was formed during the experiment, i. e. during the time the fish lived in water containing labelled phosphate.

Summary.

Sticklebacks (*Gasterosteus aculeatus*) were kept for periods of up to one month in 3 litres of sea-water containing labelled phosphate. A fish weighing 1 g was found to take up in the course of 16 days $\frac{1}{40{,}000}$ part of the phosphorus present in the water, corresponding to $2 \cdot 10^{-5}$ mg P.

By comparing the specific activity of the skeleton P with that of the free liver P, figures for the degree of the renewal of the skeleton were obtained. At least 95 per cent of the skeleton were found to remain unchanged during the experiment. The rate of renewal of the fish skeleton is thus much lower than that of the mammalian skeleton.

The specific activity of the egg P being only slightly lower than that of the free liver P, we may conclude that the greater part of the P atoms present in the eggs was incorporated during the experiment.

The writer wishes to express his cordial thanks to the trustees of the Kristineberg Zoological Station, especially Professor JOHN RUNNSTRÖM and to the Director of the Station, Dr. GUNNAR GUSTAVSON, for their unfailing kindness and support.

Stockholm, October 1944.

References.

HEVESY, G., and L. HAHN, Det Kgl. Danske Vid. Selsk. Biol. Medd. 1938. XIV. 2.
HEVESY, G., H. LEVI and O. REBBE, Biochem. J. 1940. *34.* 532.
HEVESY, G. and O. REBBE, Acta Physiol. Scand. 1940. *1.* 171.
KJERULF-JENSEN, K. and E. LUNDSGAARD, Ibidem 1943. *7.* 209.
KROGH, A., Osmotic Regulation in Aquatic Animals. Cambridge 1939.
MANLY, R. S., H. C. HODGE and M. LE FEVRE MANLY, J. Biol. Chem. 1940. *134.* 293.
ROBINSON, R., The significance of phosphorus esters in metabolism. New York 1912.
ROCHE, I. and M. MOURGUE, Bull. Soc. Chim. Biol. Paris 1939. *21.* 143.
ROTH, F., Anatom. Anz. 1920. *52.* 513.
SMITH, H. W., Amer. J. Physiol. 1930. *93.* 480. 1931. *98.* 269.

From the Departments of Medical Chemistry, Karolinska institutet, Stockholm, and the University of Uppsala, Uppsala.

The Purification and Properties of Aminopolypeptidase from the Hog's Pyloric Mucosa. I.

By

GUNNAR ÅGREN.

Received 13 November 1944.

In a previous paper (ÅGREN and HAMMARSTEN, 1937) it was demonstrated that crystallized secretin was digested by aminopolypeptidase without any loss of activity and in the process ten amino acids were split off. However, the experiments were carried out only on a very small scale with samples of about 10 mg of secretin and no attempt was made to isolate and determine the amino acid composition of the remaining part of the original secretin molecule. This problem offers several interesting aspects. The eventual presence and nature of an active group or groups in the secretin molecule would more easily be studied on the new smaller molecule. It would also be of interest to investigate if this new secretin had the same range of acitivity as the original hormone, i. e. to stimulate the secretions from the pancreas, the liver and the pyloric-duodenal mucosa.

In the planned isolation of the digested secretin molecule it was necessary to work with a more purified enzyme than in the first experiments where a very impure aminopolypeptidase only free from trypsin was used. This enzyme was prepared from the hog's pyloric mucosa (ÅGREN, 1937). At that time no successful attempt to purify the enzyme had been made and therefore the present investigation was undertaken. Already at the beginning of the purification it was found that one of the main problems was to decide whether the fairly high content of mucoids present was an integrating part of the enzyme. No data could be found of the carbohydrate content of aminopolypeptidase purified from other materials. As a preliminary study the aminopolypeptidase of

muscle was first prepared in a state of high purity and found to be a carbohydrate-free protein (ÅGREN, 1944). Accordingly it was assumed that the corresponding enzyme from the pyloric mucosa had similar chemical properties.

Experimental Procedures.

The enzyme activity was determined according to LINDERSTRÖM-LANG and HOLTER (1931, 1932) using 0.2 M solutions of alanyl-glycyl-glycine at pH 7.4 as substrate. The peptide was prepared according to FISCHER (1906, 1923) (ÅGREN, 1942). One unit of activity was defined as the amount which, after 30 minutes of digestion at pH 7.4 and 37°, gave an increase in amino nitrogen corresponding to 1 mm^3 of n/20 HCl in 90 per cent alcohol. Protein nitrogen was determined by the micro-Kjeldahl procedure. Traces of ammonium sulphate contaminating the solutions in some steps of the purification necessitated a preliminary precipitation of the protein with trichloracetic acid. 1 ml of solution containing more than 0.2 mg of nitrogen was precipitated with 2 ml of 10 % trichloracetic acid as previously described. It was necessary to keep the concentration of protein nitrogen above the value 0.2 mg/ml or else great losses were registered at the filtration of the trichloracetic acid precipitate, which at concentrations less than 0.1 mg N/ml partially precipitated in a colloidal state. Total carbohydrates were estimated by the method of TILLMAN-PHILLIPI in the modification of SÖRENSEN and HAUGAARD (1933).

Purification Procedures.

As a preliminary step to the purification of the aminopolypeptidase it was necessary to investigate the stability of the enzyme at different pH and different other experimental conditions, as cataphoresis and dialysis. Some of the results obtained from these investigations are briefly reported.

At first the enzyme solutions were prepared in the same way as previously described (ÅGREN, 1937) by grinding the fresh pyloric mucosa of the pig and extracting with equal parts of glycerol and water by shaking for 2 hours at room temperature. After the centrifugation the solution was stored at — 10° where the activity, 3.3 units per mm^3 of extract, kept constant at least for several months. In Table 1 the pH-stability of the enzyme at 0° is given. After the incubation times, 48 hours, the solutions were brought to pH 7.4 and the activity determined.

During the dialysis at pH 4.6 and 5.1 for 24 hours at 0° against glycerol-buffers a precipitation was formed in all experiments and

Table 1.

The pH stability of glycerol solutions of aminopolypeptidase stored at 0° for 48 hours at different pH.

Activity in units per mm³ solution at		
pH 4.2	pH 4.6	pH 5.1
0	3.1	3.2

separated away as it only contained traces of activity. While the activity per mg of nitrogen in the original extracts was 340 units the centrifuged pH 4.6 and 5.1 solutions contained respectively 710 and 370 units. There was the possibility that the presence of an active group in the enzyme would be unveiled by cataphorizing the enzyme at a suitable pH. In the experiments the analytical apparatus of THEORELL (1936) was used. By adding glucose in substance to the glycerol enzyme solutions it was easy to keep the specific weight values of the dialyzed solutions about 2 per cent higher than those of the buffers and thus minimize the risk of convections during the cataphoresis. The analyses were carried out on enzyme solutions purified from about 50 per cent of impurities by dialysis at pH 4.6 as described above and diluted 1: 10 with suitable glycerol buffers. In Table 2 some experimental figures are given.

Table 2.

Cataphoresis of a glycerol solution of enzyme in glycerol buffers at 0.3° C.

Glycerol concentration of 20 vol. p.c. in all tests.

Buffer	pH	$\chi \times 10^3$	i	t	Migration	v
Phosphate	6.75	0.85	3.5	21600	Anodic	0.5
Acetate	4.60	0.84	3.5	36000	0	0

χ = conductivity in reciprocal ohms; i = the current in milliamperes; t = time in seconds; v = cm migration.

The ionic velocity was not calculated since the enzyme solutions must be considered far from homogeneous. Similar qualitative results were obtained when the original unpurified enzyme solutions were cataphorized. The contents of the different anodic and cathodic cells were mixed in possible combinations but no reproducible separation of the enzyme in coenzyme and apoen-

zyme — as demonstrated by inactivation and subsequent reactivation of the enzyme activity — was obtained.

More positive evidence was obtained from some series of dialysing experiments. The enzyme solutions used in these experiments were prepared in a somewhat different manner. The distal part of pylorus and the proximal part of duodenum from hog was turned inside out, the mucosa cleaned and washed with water and the whole sample stored at —15° for some hours and thawed over night at 5°. The fluid which exuded contained aminopolypeptidase in a rather high concentration, 3—10 units per mm³ solution. One of the difficulties encountered in the dialysing experiments is demonstrated in the following. 10 ml samples of enzyme solutions, containing 3 units of activity per mm³, were dialyzed in cellophane bags, $\sigma = 27$ mm, at 0° for 24 hours during constant stirring against 1 500 ml of different acetate buffers, normality about 0.003. At the end of the dialysis the dialysates were concentrated in vacuum at 15° to the same volume as the contents of the dialysing tubes, which were centrifuged from a mostly inactive precipitate formed during the dialysis. All solutions were neutralized to pH 7.4 and the enzyme activities of the dialysates and contents of the cellophane bags were determined. Typical values are given in Table 3.

Table 3.

The activity of aminopolypeptidase dialyzed at different pH.

Activity by digestion of 7 mm³ of enzyme with 7 mm³ of substrate at 40°. Original activity in mm³ n/20 HCl, 30 minutes of digestion = 20. C = content of the dialysing tube. D = Dialysate. Experiments performed July 28th 1937.

Dialysis No.	pH at the end of dialysis	Enzyme activity in mm³ n/20 HCl	Digestion time in minutes
XI C	3.75	0	30
XI D	3.80	0	120
XII C	4.35	2.2	30
XII D	4.25	0	120
XIII C	4.75	18	30
XIII D	4.80	0	120

Buffer in XI: 5 ml n/1 Acetic acid+0.30 ml n/1 Sodium acetate diluted to 1500 ml
» » XII: 3.5 ml » » +1.5 » » » » » » » »
» » XIII: 2.5 » » » +3.0 » » » » » » » »

Dialysing at pH 3.8 and 4.3 obviously inactivated the enzyme while the activity was not affected by dialysing at pH 4.8. The following day equal volumes of the dialysates and contents of the

dialysing tubes were mixed and digested for 30 minutes with the following results. (Table 4.)

Table 4.

Incubation of dialyzed solutions of aminopolypeptidase with dialysates.

Experimental data as in Table 3. Activity per 7 mm³ of enzyme.

Samples No.	Fractions	Digestion time in minutes	Experiments performed	Enzyme activity in mm³ of n/20 HCl
1	XI C+XII D	30	July 29th	0.5
2	XI C+XIII D	»	»	0
3	XII C+XII D	»	»	2.3
4	XI C	»	July 30th	1.0
5	XII C	»	»	4.0
6	XII C+XII D	»	»	1.8
7	XI C+XI D	»	July 31th	1.4
8	XI C	»	August 2nd	2.4
9	XII C	»	»	5.2

Comparing the enzyme values of samples No. 1 and 3, Table 4, with the values of XI c and XII c, Table 3, it would at first seem as if a partially reactivation had taken place. What actually happens is that the dialyzed and partially inactivated enzyme spontaneously is reactivated. The process seems to be rather slow (compare XI c and XII c, Table 3, with samples No. 4, 5, 8 and 9, Table 4).

Next some series of dialysing experiments were performed at pH 3.8 and 4.3 with enzyme solutions which had previously been dialyzed at pH 4.8. 100 ml of enzyme solution containing 6 units per mm³ of solution were dialyzed at pH 4.8 and 0° for 24 hours (Dialysis XIV). A precipitate formed during the dialysis was centrifuged off. The enzyme activity was unimpaired by the dialysis. The dialysate was concentrated in vacuum to the same volume as the dialyzed enzyme solution. 10 ml portions were then dialyzed at 0° and pH 3.8 respectively 4.3 for 24 hours as described before. All solutions were then neutralized to pH 7.4. The dialysates were concentrated to the same volume as the contents of the cellophane tubes and the enzyme activity was determined. Typical values are given in Table 5.

From the figures in Table 5 it is obvious that a reactivation takes place when the partially inactivated enzyme from the cellophane tubes is diluted with an equal volume of dialysate. No spontaneous reactivations were observed in these series of experiments.

Table 5.

Reactivation of aminopolypeptidase inactivated by dialysis at pH 5.8 and 4.3.

Experimental data as in Table 3.

No.	Fraction	Digestion time in minutes	Date	pH at the end of dialysis	Enzyme activity in mm^3 n/20 HCl
1....	XV C	120	August 8th	3.90	3.5
2....	XV D	240	»	3.80	0
3....	XVI C	30	»	4.30	7.0
4....	XVI D	240	»	4.30	0
5....	XV C+XV D	120	»		7.5
6....	XVI C+XIV D	30	»		5.0
7....	XVI C+XVI D	30	»		6.0
8....	XV C	120	August 9th		3.5
9....	XVI C	30	»		6.8
10....	XV C+XIV D	120	»		9.0
11....	XVI C+XVI D	30	»		9.1

Discussion.

The cataphoretical analysis of aminopolypeptidase was carried out on rather unpure material. If any conclusions should be drawn from the results, the enzyme seems to migrate to the anode at physiological hydrogen ion concentration and come to a standstill at pH 4.6. Similar qualitative results were obtained in a cataphoretical analysis of a highly purified aminopolypeptidase from cattle muscle (ÅGREN, 1944). This supports the validity of the results obtained above. In contrast to the results obtained 1937 by ÅGREN and HAMMARSTEN on carboxypolypeptidase, aminopolypeptidase migrated in glycerol solutions where the latter enzyme obviously is dissociated.

The results obtained in the dialysing experiments are rather interesting. They seem to demonstrate the presence of a low molecular activator in aminopolypeptidase. The nature of the group has as yet not been elucidated. From the work of MASCHMAN (1943) it is clear that peptidases of bacterial origin inactivated by precipitation with acetone can be reactivated by addition of magnesium and mangan ions. In a recent paper SMITH and BERGMANN (1944) also demonstrated that dipeptidases, especially leucinpeptidases from the hog's duodenal mucosa, after inactivation by precipitation with ammonium sulphate or acetone could be reactivated by addition of Mn^{++} or Mg^{++}. These authors also investi-

gated enzymes from the duodenal mucosa splitting tripeptides as alanylglycylglycine, leucylglycylglycine and several tripeptides containing diglycine. There was no activation by metal ions. The question if the active group in aminopolypeptidase is a metal must at present be left open. From the work of SMITH and BERGMANN it is also clear that the problem can be advantageously studied only on the purified enzyme.

Summary.

In cataphoretical analysis the aminopolypeptidase of the hog's pyloric-duodenal mucosa behaves as a compound with its isoelectric point close to pH 4.6. After inactivation by dialysis at pH 3.8—4.3 the enzyme can be reactivated by addition of a dialysable factor.

The writer is indebted to the Johan and Therese Anderssons Minne foundation for grants which supported the present investigation. He further acknowledges the valuable assistance of Mr L. ÅGREN in carrying out some of the enzyme determinations.

References.

FISCHER, E., Untersuchungen über Aminosäuren, Polypeptide und Proteine. Berlin 1906 and 1923.
LINDERSTRÖM-LANG, K., and H. HOLTER, Hoppe Seyl. Z. 1931. *201.* 9.
LINDERSTRÖM-LANG, K., Ibidem 1932. *204.* 15.
MASCHMANN, E., Erg. Enzymf. 1943. *9.* 155.
SMITH, E. L., and M. BERGMANN, J. biol. chem. 1944. *153.* 627.
SÖRENSEN, M., and G. HAUGAARD, C. R. lab. Carlsberg Sér. chim., 1933. *19.* N:o 12.
THEORELL, H., Hand. Biol. Arb.Meth. 1936. *10.* 1083.
ÅGREN, G., Hoppe Seyl. Z. 1937. *246.* 280.
ÅGREN, G., Ark. Kemi, Mineral. Geol. 1942. *16 B.* No. 6.
ÅGREN, G., This journal 1944. *8.* 369.
ÅGREN, G., and E. HAMMARSTEN, J. Physiol. 1937. *90.* 330.

From the Departments of Medical Chemistry at Karolinska Institutet, Stockholm, and the University of Uppsala, Uppsala.

The Purification of Aminopolypeptidase from the Hog's Pyloric Mucosa. II.

By

GUNNAR ÅGREN.

Received 13 November 1944.

The aim of the present investigation, started in 1937, was to purify the aminopolypeptidase from the hog's pyloric mucosa which according to previous investigations contains large amounts of the enzyme usually not contaminated by tryptic enzymes (ÅGREN, 1937). Chemical and enzymatical methods were described in a previous paper (ÅGREN 1945). Alanyl-glycyl-glycine was used as substrate.

Purification Procedure.

Fractionated acetone precipitation of glycerol extracted enzyme.

Glycerol extracts of the mucosa as previously described (ÅGREN, 1944 a) were fractionated with ammonium sulphate and magnesium sulphate without success. At pH 7.4 magnesium sulphate did not precipitate the activity, and fractionated precipitation yielded no essential gain in purity. Several attempts to purify the enzyme by ammonium sulphate precipitation in a more acid reaction led to a complete inactivation of the enzyme. Therefore purification through acetone precipitation was tried. Table 1 demonstrates the results obtained by precipitating 10 ml of cooled glycerol extract — diluted 1: 5 with water — with acetone cooled to —80°.

Table 1.

Acetone fractionation of a solution of aminopolypeptidase.

10 ml of cooled solution precipitated by the addition of cooled acetone. The precipitates solved in 3 ml of water.

Final concentration of acetone	Protein nitrogen in mg per ml	Activity found in precipitate Units $\times 10^3$ per 3 ml	Activity calculated with 100 per cent yield Units $\times 10^3$ per 3 ml
33 %	0.31	0.57	5.1
50 %	0.33	1.41	5.1
66 %	0.33	1.86	5.1
80 %	0.36	2.80	5.1

By precipitation with acetone to 80 % a precipitate was obtained which contained 2.3×10^3 units per mg nitrogen. The glycerol solution contained 0.65×10^3 units per mg nitrogen, thus, a purification of four times was obtained, but about 50 % of the total activity was lost. Next, a series of fractionated acetone precipitations were carried out. Preliminary tests showed that the fraction precipitating between 50—80 % of acetone was the most active. In Table 2 the difference between the use of acetone cooled to —13° and —80° respectively is demonstrated.

Table 2.

Fractionated acetone precipitation of a solution of aminopolypeptidase.

20 ml cooled extract diluted 1 : 10 and precipitated by addition of acetone cooled to —13° respectively —80°. Precipitations solved on 3 ml of water.

Acetone fraction	Protein nitrogen in mg per ml	Activity found in precipitates Units $\times 10^3$ per 3 ml	Activity calculated with 100 per cent yield Units $\times 10^3$ per 3 ml
50% (—13°)	0.67	1.47	10.2
50% (—80°)	0.41	3.80	10.2
50—80% (—13°)	0.07	2.34	10.2
50—80% (—80°)	0.15	4.70	10.2

The fraction 50—80 % (—80°) gave a precipitation containing 10.4×10^3 units per mg nitrogen. The preparation had accordingly been purified about 15 times. The total yield was about 50 %. When an attempt was made to repeat the procedure with larger samples of solution (400 ml) the average total yield was only 10—15 % and the activity was about 5×10^3 units per mg nitrogen. Obviously, the extended time of handling, necessary when prepar-

ing greater volumes, had a deleterious effect on the activity. The method had to be abandoned, in particular because the preparation obtained could not be stored without constant loss of activity.

Purification of aminopolypeptidase by $(NH_4)_2SO_4$-extraction of fresh pyloric mucosa.

By extracting the pyloric mucosa with ammonium sulphate solutions, extracts suitable for further purification were obtained. Fresh, ground mucosa was extracted with four times its weight of 0.45 saturated ammonium sulphate in 0.1 m sodium bicarbonate by shaking for 1 hour at room temperature. Insoluble material was separated by centrifugation for 30 minutes at 3 000 r.p.m. The centrifugate was further purified by fractionated ammonium sulphate precipitation. To the 0.45 saturated extract, which contained about 9×10^3 units per mg nitrogen, solid ammonium sulphate was added to 0.7, 0.8, 0.9 and 1.0 saturation. The precipitates were filtered on Buchner funnels with a thin layer of coarse (Hyflow) Cel. The filter cake was extracted with 0.04 M $NaHCO_3$ and dialyzed against 0.04 M $NaHCO_3$ for 24 hours at 0°. The filtrate from the precipitation was dialyzed in the same way. In Table 3 the analyses from a series of fractionations are given.

Table 3.

Ammonium sulphate fractionation of a 0.45 saturated extract of pyloric mucosa.

Concentration of ammonium sulphate	Activity in units $\times 10^3$ per mg of nitrogen		Per cent activity of original in	
	Precipitate	Filtrate	Precipitate	Filtrate
0.45 saturated extract		9.0		
0.7 — saturation	8.6	2.4	10	90
0.8 — saturation	7.5	4.0	25	75
0.9 — saturation	12.1	5.0	70	30
1.0 — saturation	9.0	0	100	0

Fractionation between 0.45 and 0.90 saturation gave the best qualitative and quantitative results. A filter cake obtained by fractionation in this way was extracted with 0.1 M phosphate buffer pH 7.4. After centrifugation of Hyflow Cel the centrifugate was brought to pH 6.0 by addition of acetate buffer, pH 4.6, and mixed with an equal volume of 1 M copper hydroxide suspension, as described by HERRIOT (1938). The mixture was stirred for 5 minutes and filtered on a Buchner funnel. The copper

hydroxide cake was extracted with a volume of 0.2 M phosphate buffer equal to the volume occupied by the protein solution just before mixing with the copper suspension. The copper hydroxide was filtered off. The filtrate contained 30×10^3 units per mg nitrogen. The yield was about 90 %. Through the procedure the enzyme had been purified about 45 times, as compared with the enzyme in the glycerol extract.

Purification of aminopolypeptidase from dried pyloric mucosa. Through the Astra Corporation, Södertälje, large quantities of vacuum-dried pyloric mucosa were obtained.[1] 1 g of the dried mucosa corresponded to about 6 g of fresh material. 100 g of dried mucosa were extracted with 2 000 ml ammonium sulphate, the concentrations ranging between 0.30—0.45 saturation. The best results were obtained by extraction for 1 hour at room temperature with the 0.35 saturated solution containing 0.1 m $NaHCO_3$. The mixture was centrifuged for 30 minutes at 3 000 r.p.m., and the centrifuged and filtered solution was brought to 0.9 saturation by adding solid ammonium sulphate. The suspension was allowed to settle for about 30 minutes, and filtered after addition of about 7 g of coarse Cel on 30 cm Buchner funnels covered with filter paper and a layer of coarse Cel. The filtrate was discarded. The filter cake was extracted with 0.1 M phosphate buffer pH 7.4 and the filter Cel separated by centrifugation. An attempt was made to repeat the copper hydroxide adsorption as described above. Qualitatively, the degree of purification was the same, but the yield was only a few per cent and could not be raised, in spite of extensive variations of the experimental conditions.

Next, an attempt was made to purify the enzyme of the filter cake from 0.9 saturation by repeated ammonium sulphate fractionations. The filter cake was extracted with a volume of 0.1 M phosphate buffer pH 7.4 equal to one third of the volume occupied by the protein solution just before bringing it to 0.9 saturation with ammonium sulphate. The filter Cel was separated by centrifugation and the centrifugate divided into equal parts and precipitated by 0.70, 0.80 and 0.90 saturation by addition of the solid salt. The activity was quantitatively recovered, but there was no essential gain in purification.

Different adsorption methods were investigated. The enzyme solution was a phosphate extract of the filter cake obtained by fractionated precipitation between 0.35 and 0.90 saturation, as

[1] Thanks are due to the Astra Corporation for gifts of Stotal powder.

described above. The solution was dialyzed at 0° for 6 hours against 0.01 M disodium phosphate. Fresh precipitated $CaCO_3$ partially adsorbed the activity, but no purification was obtained. On freshly precipitated $Ca(OH)_2$ the enzyme was completely adsorbed, but could not be profitably eluated. The Tswett adsorption procedure was next investigated. A column of $CaCO_3$ washed with acetate buffer pH 5.6 was easily passed by the enzyme, as well as by the nitrogen and carbohydrate impurities. On a column of Brockmann Al_2O_3 the enzyme was strongly adsorbed, as demonstrated by the following experiment.

20 ml of a dialyzed extract on the filter cake obtained by fractionated precipitation between 0.35 and 0.9 saturation, as described above, was passed through a 6 cm high column of closely packed Brockmann Al_2O_3. The column had previously been washed with 20 ml of 0.1 N acetate buffer pH 5.7 and the enzyme solution had been acidified to the same pH. The column was afterwards washed with 20 ml of 0.1 N acetate buffer pH 5.7, which were taken together with the filtered enzyme solution. The column was divided into three equal parts. Each was eluated with 10 ml of 0.1 M phosphatebuffer pH 7.8. The results of the chemical and enzymatical analysis are given in Table 4.

Table 4.

Adsorption of a solution of aminopolypeptidase on Brockmann aluminium oxide.

Sample	Activity in units per mm³	Protein nitrogen in mg per ml	Total carbohydrates in mg per ml
Enzyme solution	11	0.95	0.46
Filtrate+washings	0	0	0.025
Eluate from upper third of the column	1.2	0.31	0.118
Eluate from center part of the column	1.3	0.22	0.11
Eluate from the lower part of the column	1.0	0.10	0.07

The Brockmann aluminium oxide could obviously not be used for a further purification of the enzyme. By a similar series of procedures aluminium silicate, aluminium oxide pure (Schering-Kahlbaum), aluminium oxide techn. (Schering-Kahlbaum), aluminium hydroxide pure (Schering-Kahlbaum), aluminium hydroxide free from alkali (Schering-Kahlbaum) and bauxite were tested without more success. Usually, the enzyme activity cal-

culated per mg nitrogen increased, but that calculated per mg carbohydrate was unchanged. The total yield of activity was usually not more than about 10 per cent. A necessary condition for reproducible results was to dialyze the enzyme solutions before the adsorption, so that the ammonium sulphate concentration was less than 1 mg per ml. Solutions containing higher concentrations of ammonium sulphate were only slightly adsorbed during the standardized conditions — 30 ml enzyme solution and a 7 cm high column of about 12 g of the aluminium compound to be tested — owing to the fact that the enzyme solution turned alkaline during the passage of the column. More promising results were obtained with aluminium oxide anhydrous extra pure (MERCK). The following solutions were used in a typical experiment:

S 1 = 1 part 0.1 N acetic acid + 9 parts of 0.1 N sodium acetate.
S 2 = 1 part 1 N acetic acid + 9 parts of 1 N sodium acetate.
S 3 = 1 part 8 N acetic acid + 9 parts of 8 N sodium acetate.
S 4 = 1 part 0.1 monosodium phosphate + 9 parts of 0.1 M disodium phosphate.
S 5 = 27 ml dialyzed extract of the filter cake from the usual ammonium sulphate fractionation. The filter cake was extracted with 0.1 M phosphate buffer pH 7.4 until no more activity could be washed out, and then dialyzed for 6 hours at 0° against 0.01 M disodium phosphate. The dialyzed enzyme solution was free from ammonium sulphate (NESSLER). 3 ml S 2 and 0.7 ml S 3 were added. The nitrogen content of S 5 should be about 0.8 mg/ml.

The column was washed by the passage of 15 ml S 1. Then S 5 was passed by gentle suction, the velocity being about 2 ml per minute, and afterwards the column was washed with 40 ml of S 1. The filtrate from S 5 and the 40 ml of S 1 (= Filtrate 1 A and 1 B) were dialyzed for 6 hours against 0.01 M disodium phosphate. The column was shaken in two periods of 30 minutes each, with respectively 30 and 20 ml of S 4. (= Filtrate 2 A and 2 B). Chemical and enzymatical analysis are given in Table 5. The aluminium oxide could not immediately be used for further experiments.

In S 5 the activity per mg nitrogen and carbohydrate were respectively 4×10^3 and 9×10^3 units. In F2A the values were respectively 8×10^3 and 20×10^3 units, and in F2B 8×10^3 and 20×10^3. If F2A and F2B were taken together the enzyme was purified twice

Table 5.

The adsorption of a solution of partially purified aminopolypeptidase on Merck's anhydrous aluminium oxide.

The abbreviations are explained in the text.

Sample	Volume in ml	Activity in units per mm³	Protein nitrogen in mg per ml	Carbohydrates in mg per ml	Yield in per cent
S 5	30	3.0	0.77	0.34	100
F 1A	30	0	0	0.04	0
F 1B	40	0.05	0.05	0.05	1.5
F 2A	30	1.8	0.22	0.09	60
F 2B	20	0.8	0.10	0.04	27

with regard to both protein and carbohydrate impurities and with a total yield of nearly 90 per cent of the original activity.

The dialyzed enzyme extract used in the previous experiment could also be purified by precipitation with lead acetate in the following way. 10 ml samples of the solution were precipitated with respectively 0.3, 0.4 and 0.5 ml of 0.5 N lead acetate. The precipitates were immediately centrifuged, the centrifugates neutralized to pH 7.4 and analyzed. Typical results are given in Table 6.

Table 6.

The precipitation of a partially purified solution of aminopolypeptidase by lead acetate.

Lead acetate in ml per 10 ml of enzyme solution	Activity in units per mm³	Protein nitrogen in mg per ml	Carbohydrates in mg per ml	Yield in per cent
Enzyme solution	5.0	0.72	0.38	100
» » +0.3 ml	3.0	0.60	0.31	60
» » +0.4 ml	2.5	0.20	0.20	50
» » +0.6 ml	2.0	0.15	0.13	40

The enzyme activity calculated per mg nitrogen and carbohydrate after precipitation with 0.3 ml lead acetate was respectively 5×10^3 and 9.5×10^3 units, with 0.4 ml lead acetate 12.5×10^3 and 12.5×10^3 units, and with 0.6 ml lead acetate 13×10^3 and 15×10^3 units. In spite of the less favourable yield it was decided to use 0.6 ml lead acetate per 10 ml extract. With a combination of lead acetate precipitation and adsorption on aluminium oxide a further purification could be obtained. The following solutions were used.

S 1 = 0.1 N acetate buffer pH 5.7
S 2 = 1 N acetate buffer pH 5.7
S 3 = 0.5 N lead acetate
S 4 = A dialyzed solution of enzyme prepared as stated above in the adsorption experiment.
S 5 = 0.1 M phosphate buffer pH 7.8.

To 50 ml S 4 were added 3 ml S 3. The precipitate was centrifuged and to the centrifugate was added 5 ml S 2 (= S 6). This solution was sucked through the standard column of anhydrous aluminium oxide previously washed with 15 ml S 1. (Filtrates respectively F1A and F1B.) The column was afterwards washed with 30 ml of S 1, and then the enzyme activity was eluated by shaking the aluminium oxide twice for 30 minutes with respectively 30 and 20 ml of S 5. Each time the aluminium oxide was filtered off. (Filtrates respectively F2A and F2B.) The filtrated eluates were analyzed after neutralization to pH 7.4. Typical values are given in Table 7.

Table 7.

The purification of aminopolypeptidase by precipitation with lead acetate followed by adsorption on anhydrous aluminium oxide. Small scale experiment.

The abbreviations are explained in the text.

Sample	Volume in ml	Activity in units per mm³	Protein nitrogen in mg per ml	Carbohydrates in mg per ml	Yield in per cent
S 4	50	3.8	0.80	0.38	100
S 6	45	1.5	0.15	0.10	36
F 1A, F 1B	80	0	0	0	0
F 2A	30	1.9	0.05	0.03	30
F 2B	20	0.5	0.02	0.01	5

The enzyme activity calculated per mg nitrogen and carbohydrate in F2A were 38×10^3 and 63×10^3 units and in F2B 25×10^3 and 50×10^3 units. The advantage of the combined method is obvious.

When an attempt was made to apply the combined method on a larger scale several difficulties were encountered. All the experiments necessary to standardize the procedure cannot be given in details. On the small column it had been tested that 60 ml of enzyme solution precipitated with lead acetate as described

above could be adsorbed without greater losses. The large column was also made 7 cm high, and contained 12 times as much Al_2O_3 as the small one. The intention was to use each column for the adsorption of 600 ml of the dialyzed extract of the filter cakes. They were prepared from 10 kg of dried pyloric mucosa in fractions of 100 g, which were extracted and fractionated between 0.35 and 0.9 saturation with ammonium sulphate, as described above. The enzyme precipitated with 0.90 saturation was stored at 0° until all the material was prepared to this stage, whereupon a thorough mixture of all the filter cakes followed. About 40 g of the mixed filter cakes were extracted with 100+80+50 ml of 0.1 M phosphate buffer pH 7.4, and dialyzed for 4 hours at 0°, stirred all the time, in three cellophane tubes $\emptyset = 27$ mm, against 5 000 ml of 0.01 M disodium phosphate, which were renewed after 60 and 150 minutes. After dialysis the content of the cellophane tubes — containing less than 1 mg of ammonium sulphate per ml — was diluted with water to about 600 ml (S1). The protein nitrogen should be about 0.8 mg per ml. The solutions in portions of 100 ml were heated at 50° for 3 minutes, and rapidly cooled to room temperature. (S 2). Then a preliminary adsorption with anhydrous Al_2O_3 (MERCK) followed. After heating the solution was acidified to pH 6.0, and 18 g of Al_2O_3 were added. The mixture was shaken for 5 minutes and centrifuged. 100 ml portions of the centrifugate (S 3) were precipitated with the optimal amount of 0.5 N lead acetate, usually 5 ml per 100 ml S 3. The lead acetate was added by drops with constant shaking, and followed by a careful grinding of the precipitate with a glass rod. The mixture was centrifuged for 5 minutes at 3 000 r.p.m. To the centrifugate (S 4) were added 150 ml 1 N acetate buffer pH 5.7, and after washing the column with 200 ml of 0.1 N acetate buffer pH 5.7 the acidified enzyme solution was sucked through the column. This was washed with 300 ml of 0.1 N acetate buffer pH 5.7 and then the column was extracted for 30 minutes with 500 ml of 0.1 M phosphate buffer pH 7.8. The mixture was filtered through a Buchner funnel and the filtrate (F 1) analyzed after neutralization to pH 7.4. In Table 8 the results from a typical experiment are given.

The enzyme activity calculated per mg of nitrogen and carbohydrate in F1 were 50×10^3 and 83×10^3 units. The corresponding figures in S 1 were 7.5×10^3 and 13×10^3 units. The following remarks may be made to the method outlined above. The dialyzed solution

must not contain more than 1 mg of ammonium sulphate per ml enzyme solution. Otherwise the lead acetate precipitation will not give optimal results and the adsorption on Al_2O_3 will be carried out in alkaline reaction. The dialyzed solution must not be stored at 0° for more than 24 hours. Through the heating procedure the enzyme activity was stabilized, so that the concentrated and precipitated enzyme in F 1 could be stored for several months without inactivation.

Table 8.

The purification of aminopolypeptidase by precipitation with lead acetate followed by adsorption on anhydrous aluminium oxide. Large scale experiment.

The abbreviations are given in the text.

Sample	Volume in ml	Activity in units per mm³	Protein nitrogen in mg per ml	Carbohydrates in mg per ml	Yield in per cent
S 1	600	6.0	0.80	0.45	100
S 2	600	5.8	0.77	0.44	97
S 3	580	5.0	0.70	0.30	80
S 4	550	4.4	0.12	0.10	61
F 1	530	2.5	0.05	0.03	37

The enzyme solution purified through the method outlined above was acidified to pH 6.6 and concentrated in a vacuum at +12° to 1/15 by volume. Phosphate crystals formed during the concentration were filtered off and the filtrate brought to 0.9 saturation of ammonium sulphate in m/10 sodium bicarbonate by adding the solid salts. The suspension mixed with some coarse Cel was filtered on a Buchner funnel covered with a layer of coarse Cel. The filtrate was discarded. The filter cakes could be stored at 0° for several months without inactivation of the enzyme activity. Several attempts to a further purification of the enzyme through repeated fractionated precipitation with ammonium sulphate or lead acetate did not meet with any marked progress.

In view of the favourable results obtained in the cataphoretical purification of aminopolypeptidase from muscle (ÅGREN, 1944) the possibilities to purify the closely allied enzyme from the pyloric mucosa by the same method were further investigated. As previously demonstrated the enzyme in an impure state seemed to have an isoelectric point close to pH 4.6 (ÅGREN, 1945). 1 g of the filter cake containing enzyme purified by the combined method was extracted with 20 ml of water and dialyzed in cello-

Table 9.

The peptidase activity of phosphate extracts of dried respectively fresh pyloric mucosa and the highly purified aminopolypeptidase obtained from the dried mucosa.

Activity calculated per 8 mm^3 of extract and 1 hour digestion at pH 7.4 and 40°.

Sample	Enzyme activity in mm^3 n/20 HCl														
	Aminopolypeptidase						Dipeptidase								
	AGG	AGA	GLG	GGG	ALG	GLA	AG	GA	LG	GG	GV	VG	AL	LA	VA
Extract of dried pyloric mucosa	68	51	24	20	19	12	10	3.0	3.2	1.5	0	3.1	0	0	0
Extract of fresh pyloric mucosa	80	69	44	25	28	23	2.0	33	4.2	21	5.5	7.8	0	0	0
Extract of purified aminopolypeptidase	70	79	47	46	6.0	7.1	0.5	0	0.2	0.4	0	0.1	0	0	0

Abbreviations: A = alanine; G = glycine; L = leucine; V = valine.

phane tubes, Ø = 27 mm, for 6 hours at 0° with constant stirring against 1 500 ml of the buffer to be used in the cataphoresis The buffer was renewed after 2 and 4 hours of dialysis, and after 6 hours the values of pH and conductivity of the buffer and the content of the cellophane tube were the same Some cataphoretical experiments were carried out at pH 6.8 and 5.8 with phosphate buffers of the usual ionic strength and the same strength of current. At both pH only one component migrated to the anode, none to the cathode. At pH 6.8 the migrating boundary had just passed out of the top anodic cell after 3 hours, and at pH 5.80 after $6^1/_2$ hours. In several experiments both at pH 6.80 and 5.80 the cataphoresis was interrupted after shorter times, and the enzyme activity of the different cells was analyzed. The enzyme activity of the top anodic cell corresponded to what would be the calculated value if the migrating boundary was caused by the migrating enzyme. At pH 5.80 the activity of the enzyme which had migrated into the top anodic cell was 60×10^3 units per mg nitrogen and 160×10^3 units per mg carbohydrate. The cataphoretical experiments could not be carried out at a pH less than 5.0, since the enzyme in the final pure state was slowly inactivated in the dialysis preceding the cataphoresis. The enzyme gave the usual

Table 10.

The purification of aminopolypeptidase.

Procedure	Activity in units $\times 10^3$ per mg protein nitrogen	Activity in units per mg carbohydrate	Yield
30% glycerol extract of pyloric mucosa	0.65	1.1	
100 g of dried pyloric mucosa extracted with 2,000 ml 0.35 saturation of ammonium sulphate in m/10 sodium bicarbonate by shaking for 1 hour. The unsolved separated by centrifugation and filtration on a Buchner funnel covered with coarse (Hyflow) Cel.=1	2.0	3.6	100
No. 1 was brought to 0.90 saturation of ammonium sulphate by addition of the solid salt. The suspension was allowed to settle for 1 hour and filtered after the addition of 7 g of coarse Cel on 30 cm Buchner funnels covered with filter paper and with a layer of coarse Cel. The filter cake was extracted with 0.1 1 phosphate pH 7.4 and dialyzed against 0.01 M disodiumphosphate. The dialyzed enzyme solution diluted to contain about 0.8 mg nitrogen per ml.=2 ..	8.4	13	80
No. 2 heated to 50° for 3 minutes and the cooled solution shaken with anhydrous Al_2O_3 for five minutes filtered and precipitated with optimal amount of 0.5 N lead acetate. Centrifugate=3	37	44	44
No. 3 adsorbed on about 100 g of anhydrous Al_2O_3 (Merck) and eluated with m/10 phosphate buffer pH 7.8 = 4	50	83	30
No. 4 concentrated to $^1/_{15}$ by volume, filtered from phosphate crystals and brought to 1.0 saturation of ammonium sulphate in m/10 $NaHCo_3$ by addition of the solid salts. About 0.5 g of coarse Cel was added and the suspension filtered on a Buchner funnel covered with a layer of coarse Cel = 5	50	83	30
About 1 g of filter cake from 5 extracted with water, dialyzed against cataphoresis buffer pH 5.8 for 6 hours. Cataphoresis for 6.5 hours. The content of the top anodic cell = 6	60	160	7

colour reactions for amino acids. Several attempts to crystallize the enzyme were carried out on the concentrated eluates from the combined lead acetate-adsorption procedure, but without success.

In a previous paper it was demonstrated that extracts from the fresh and dried pyloric mucosa had about the same range of enzyme activity when tested on a series of peptides (ÅGREN, 1942). For purposes of comparison a similar investigation was carried out with the highly purified aminopolypeptidase (cataphorized material). The values are given in Table 9.

The most conspicuous result is that the purified aminopolypeptidase has been almost completely freed from the dipeptidase activity present in the original enzyme extract. The enzyme affinity against the different tripeptides had not been much changed during the purification. Quantitative data from the different stages of the purification method are given in Table 10.

Discussion. By the method outlined above the aminopolypeptidase of the pyloric mucosa can be purified about 100 times, when calculated on nitrogen values, and obtained in a fairly pure state. The yield of the method was about 30 per cent. The purest product obtained by cataphoretical analysis contained less than 3 per cent of carbohydrate, and the enzyme is probably a carbohydrate-free protein like the closely allied enzyme present in the muscle (ÅGREN, 1944). The purified enzyme was practically free from the dipeptidases present in the original extract. The cataphoretical homogeneity does not, however, prove that a single enzyme is responsible for the splitting of all the analyzed tripeptides. On the other hand, the tendency of the enzyme to split the different tripeptides is about the same in the crude original extract and in the final purified product. That must mean that if we are not dealing with a single enzyme the different enzymes must be closely allied chemically, in order to run parallel through the whole purification method. The instability of the purified enzyme did not allow of a more accurate determination of the isoelectric point, but the data available does not contradict the preliminary results showing that the isoelectric point would be close to pH 4.6. Several experiments were also performed on the purified enzyme to settle the question, discussed in a previous paper, as to whether aminopolypeptidase is a manganese protein. The enzyme was inactivated by different procedures, but no activation of added Mn++ was obtained. At the time of the investigation (the beginning of 1942) the purification method outlined above had to be abandoned due to the lack of the special aluminium oxide used, during prevailing conditions.

Summary.

The aminopolypeptidase from the hog's pyloric mucosa has been purified about 100 times and obtained in a cataphoretical homogeneous state. The enzyme is practically free from dipeptidase activity and seems to be a protein, free from, or with a very low content of, carbohydrates.

The writer is indebted to the Johan and Therese Anderssons Minne Foundation, the Karl Petrén Foundation and the Astra Corporation for grants which supported the present investigation. He further acknowledges the valuable assistance of Messrs L. ÅGREN and SCHEIN as well as of Mrs ÅGREN and KARLSSON, and of Miss SUNDQUIST throughout the investigation.

References.

HERRIOT, R. M., J. Gen. Physiol. 1938. *21*. 501.
ÅGREN, G., Hoppe Seyl. Z. 1937. *246*. 280.
ÅGREN, G., This journal 1944. *8*. 369.
ÅGREN, G., Ibidem 1945. In press.

From the Departments of Physiological Chemistry at Wenner-Gren's institut, Stockholm, and the University of Uppsala, Uppsala.

The Purification of Aminopolypeptidase from the Hog's pyloric Mucosa. III.

By

GUNNAR ÅGREN.

Received 13 November 1944.

In a previous paper a method was described to purify the aminopolypeptidase from the hog's pyloric mucosa 100 times (ÅGREN, 1945 a). In the beginning the interest was focussed mainly on the preparative work. However, the question came to the fore as to whether aminopolypeptidase could be identical with Castle's intrinsic factor (ÅGREN, 1942). In a preliminary investigation with partially purified material (purified about 10 times) it was found that the enzyme solution apparently contained most of the intrinsic factor activity present in the mucous membrane. It was therefore of interest to determine the intrinsic factor activity of the highly purified aminopolypeptidase. The previously described method could not be followed since the anhydrous aluminium oxide used there was not available, due to prevailing conditions. This paper describes a partially new method, which gives a preparation of at least the same purity. Chemical and enzymatical methods were described in a previous paper (ÅGREN, 1945 b).

Purification procedure. First of all, an attempt was made to regenerate the anhydrous aluminium oxide, used during the work by the previous method, with washing and boiling the product for different times with acids of different strength. The best results were obtained by shaking for 30 minutes with 0.5 N HCl and then washing with distilled water. The yield of activity when using the regenerated Al_2O_3 was low however, even if the purity of product was satisfactory. Kaolin treated in different ways could not be used for adsorption. An attempt was also made

using a solution purified by lead acetate precipitation, as described in the previous method, for the cataphoretic separation of the impurities without preliminary treatment with anhydrous aluminium oxide. Experiments were carried out at pH 5.5 in phosphate buffer and at pH 4.2 in acetate buffer of about the same ionic strength and the same strength of current. At pH 5.5 the enzyme migrated anodically and filled the top anodic cell after about 7 hours. The enzyme activity in the content of this cell was calculated per mg nitrogen and carbohydrate 45×10^3 and 120×10^3 units respectively. The corresponding figures of the solution precipitated with lead acetate were 24×10^3 and 61×10^3 units respectively. The purification was good, but the enzyme was purified about 70 times instead of 100 times. At pH 4.2 only one cathodically migrating boundary could be observed. After 3 hours it had passed about $^1/_3$ of the top cathodic cell. Next, we tried to treat the filter cake, obtained by fractionated purification of the enzyme between 0.35 and 0.90 saturation of ammonium sulphate, with a saturated solution of sodium chloride, ammonium sulphate and sodium bicarbonate. The solution was made by shaking a saturated solution of sodium chloride with a surplus of the other solid salts, and had successfully been applied by HAMMARSTEN in the separation of nucleic acids from proteins. By this procedure about half of the carbohydrate impurities in the enzyme precipitated with 0.90 saturation of ammonium sulphate could be removed with only a 4 % loss of the enzyme activity. When the residue was extracted with 0.1 M phosphate buffer, dialyzed and precipitated with the optimal amounts of lead acetate, as described in the previous paper, the activity calculated per mg of carbohydrate was not higher than when a phosphate extract from the untreated filter cake was precipitated with lead acetate under optimal conditions. These experiments, however, gave the valuable information that the carbohydrate impurities were more soluble than the enzyme activity in high concentrations of ammonium sulphate.

Accordingly, a further purification was obtained by fractionated precipitation with ammonium sulphate of the enzyme solution precipitated with lead acetate as described in the previous paper. The method finally adopted was the following.

1). 100 gm of vacuum dried pyloric mucosa were extracted with 2 000 ml of a 0.35 saturated solution of ammonium sulphate in 0.1 M sodium bicarbonate by shaking for 1 hour. The unsolved

material was removed by centrifugation for 30 minutes at 3 000 r. p. m. To the centrifugate about 7 gm of coarse (Hyflow) Cel were added, and the suspension filtered on 30 cm Buchner funnels covered with filter paper and a layer af coarse Cel. The filtrate was brought to 0.9 saturation by the addition of solid ammonium sulphate. The suspension was allowed to stand for about 30 minutes and, after the addition of about 7 gm of coarse Cel, filtered as just described. The filter cake containing the enzyme was easily removed from the rest of the Hyflow layer. About 10 kg of dried pyloric mucosa was prepared to this stage and stored at 0°.

2). From about 160 gm of the filter cake the enzyme activity was quantitatively removed by extraction 3 times with 400, 320 and 200 ml of 0.1 M phosphate buffer, pH 7.4 and centrifugation of the filter Cel. The centrifugate was dialyzed in 9 cellophane tubes, $\varnothing = 27$ mm, at 0° for about 4 hours with constant stirring against 0.01 M disodium phosphate, which was renewed after 60 and 150 minutes. The content of each cellophane tube was in this way dialyzed against 5 000 ml of 0.01 M disodium phosphate. The dialyzed enzyme solution was diluted with water to 2 400 ml. The protein nitrogen in the diluted solution should amount to about 0.8 mg per ml. The solution in portions of 200 ml was rapidly brought to 50° and kept at that temperature for 3 minutes, then rapidly cooled to room temperature again.

3). The heated enzyme solution was precipitated in 100 ml portions with the optimal amount of 0.5 N lead acetate, usually 5 ml per 100 ml enzyme solution. The lead acetate was added by drops with constant shaking, and followed by a careful grinding of the precipitate with a glass rod. The mixture was centrifuged for 6 minutes at 3 000 p. r. m. The centrifugate was neutralized to pH 7.4, and separated or filtered from an opalescense which developed at the neutralization.

4). The clear solution was concentrated in a vacuum at 12° to a tenth of the original volume and brought to 0.8 saturation of ammonium sulphate in 0.1 M sodium bicarbonate by adding the solid salts. Some coarse Cel was added and the suspension filtered on a Buchner funnel covered with filter Cel. The filter cake containing the enzyme was easily removed from the rest of the Hyflow layer. All the enzyme material stored as filter cake in 1) was prepared up to this point and stored at 0°.

5). From a filter cake of 4) the enzyme was quantitatively re-

moved by extraction 4 times with 15+10+10+5 ml of 0.1 M phosphate buffer pH 7.4. Each time the filter Cel was separated by centrifugation for 20 minutes at 15 000 r. p. m. The centrifugate was dialyzed for 2 hours against 0.01 M disodium phosphate as described in 2), the phosphate solution being renewed after 60 minutes. The dialyzed enzyme solution was brought to 0.6 saturation of ammonium sulphate in 0.1 M sodium bicarbonate by the addition of the solid salts. Some filter Cel was added and the suspension filtered on a Buchner funnel. The enzyme material stored at 0° in 4) was prepared to this stage and stored at 0°.

6). From a filter cake of 5) the enzyme was extracted and dialyzed as in 5). The dialyzed solution was brought to 0.5 saturation of ammonium sulphate in 0.1 M solution of sodium bicarbonate by adding the solid salts. The precipitate was centrifuged for 20 minutes at 15 000 r. p. m. The centrifugate was brought to 0.55 saturation of ammonium sulphate and centrifuged in the same way. The centrifugate was precipitated at 0.6 and then at 0.7 saturation of ammonium sulphate. The precipitates were solved in water. In Table 1 the chemical and enzymatical analyses of a typical fractionation are given.

Table 1.

Fractionation of a purified enzyme solution with ammonium sulphate of 0.50 up to 0.70 saturation.

Precipitate from	Solved to	Enzyme activity in units × 10^3 per ml solution	Protein nitrogen in mg per ml	Carbohydrates in mg per ml
0.5 saturation.....	5 ml	220	1.9	1.0
0.55 saturation.....	8 »	600	4.9	2.9
0.60 saturation.....	14 »	86	6.3	7.5
0.70 saturation.....	8 »	8.6	1.0	0.8
Original solution	40 »	183	5.4	4.0

The activity calculated per mg of nitrogen and carbohydrate of the 0.50 fraction were 115 × 10^3 and 220 × 10^3 units, and the corresponding figures for the 0.55 fraction were 120× 10^3 and 210 × 10^3 units. These two fractions were taken together, and the solutions stored at —20°. The intrinsic factor activity of a solution prepared according to the method outlined above has been previously reported (ÅGREN and WALDENSTRÖM, 1944). Quantitative data of the different stages of the method are given in Table 3.

Table 2.

The purification of aminopolypeptidase.

The numbers refer to the figures of the different stages of the method of preparation given in the text	Activity in units $\times 10^3$ per mg protein nitrogen	Activity in units $\times 10^3$ per mg carbohydrate	Yield in per cent
30% glycerol extract of pylorie mucosa	0.65	1.1	
No. 1	2.0	3.6	100
No. 2	7.8	14	80
No. 3	17.1	18	40
No. 4	25	28	37
No. 5	34	45	37
No. 6	115	210	30

The question as to whether aminopolypeptidase is a metal protein was investigated, by using the purified enzyme solution from 6). The presence of vitamin C in a concentration of 0.01 M did not influence the activity of the enzyme. Copper ions inactivated the enzyme, as demonstrated by the following series:

Activity of the enzyme solution (control) in units per mm^3=1.

Activity in the presence of 0.0025 M copper acetate in units per mm^3 = 0.23.

Activity in the presence of 0.0012 M copper acetate in units per mm^3 = 0.26.

Activity in the presence of 0.00025 M copper acetate in units per mm^3 = 0.43.

Activity in the presence of 0.00012 M copper acetate in units per mm^3 = 0.59.

Cysteine and pyrophosphate also inactivated the enzyme:

Activity in the presence of 0.01 M cysteine in units per mm^3 = 0.40.

Activity in the presence of 0.001 M cysteine in units per mm^3 = 0.67.

Activity in the presence of 0.001 M pyrophosphate in units per mm^3 = 0.47.

NaCN in a concentration of 0.01 N did not inactivate the enzyme, but BERGMANN and FRUTON (1937) reported that 0.1 N cyanide partially inactivated crude solutions of aminopolypeptidase. The inhibiting effect of Cu^{++} could be partially re-

versed by the addition of NaCN, as demonstrated by the following series:

1). Activity in the presence of 0.0012 N copper acetate in units per $mm^3 = 0.17$.

2). Activity in the presence CN^- 0.012 N and Cu^{++} 0.0012 M in units per $mm^3 = 0.50$.

3). Activity in the presence Cu^{++} 0.0012 M and CN^- 0.012 N in units per $mm^3 = 0.17$.

There was a tendency to formation of an opalescence in 1). No 2 was clear and in 3) the tendency to opalescence disappeared on the addition of sodium cyanide.

Discussion. By the method outlined above the aminopolypeptidase of the hog's pyloric mucosa has been purified more than 100 times when calculated on nitrogen or carbohydrate values. The yield of the method was about 30 per cent. The carbohydrate content of the purest preparation was less than 10 per cent. As compared with the previous method (ÅGREN, 1945 a) the present one is less complicated, since it does not include cataphoresis; moreover, the yield is better. The success of the present method mainly depended on the property of being soluble in rather high concentrations of ammonium sulphate, which was shown by the carbohydrate impurities left after lead acetate precipitation. The cataphoretical data are additional proof of the position of the isoelectric point of the enzyme in the vicinity of pH 4.6.

The question as to whether aminopolypeptidase is a metal protein may be somewhat discussed. In a recent paper (ÅGREN, 1945 b) it was demonstrated that the enzyme after inactivation by dialysis at pH between pH 3.8 and 4.2 was partially reactivated by the addition of the concentrated dialysate. The activator must accordingly be a low molecular compound. On the alkaline side of the isoelectric point, which is probably close to pH 4.6, the enzyme could only very slowly be inactivated by dialysis, and the active group seemed to be more closely attached to the protein. Inactivation of an enzyme by complexforming compounds such as cyanide, pyrophosphate and cysteine is often explained by the presence of a heavy metal as active group in the enzyme (WARBURG and CHRISTIAN, 1943). This may also be the explanation of the inhibiting effects of these compounds on aminopolypeptidase activity, especially since the activator must be a low molecular substance as described above. Mn^{++} seems

not to be an activator, and it has not been possible to demonstrate the presence of manganese in the enzyme. The nature of the activator will be further investigated. At the time of the investigation (the beginning of 1943) the purification method outlined above also had to be abandoned, owing to the lack of the coarse (Hyflow) Cel under prevailing conditions.

Summary. A new method has been described for the purification 100 times of aminopolypeptidase from the hog's pyloric mucosa. The carbohydrate content of the enzyme was low. The presence and nature of the active group of the enzyme has been discussed.

The writer is indebted to the Johan Anderssons Minne Foundation, the Karl Petrén Foundation and the Astra Corporation for grants which supported the present investigation. He further acknowledges the valuable assistance of Messrs. L. Ågren and Schein, as well as of Mrs. Ågren and Mrs. Karlsson and of Miss Sundquist throughout the investigation.

References.

Bergmann, M., and J. S. Fruton, J. Biol. Chem., 1937. *117.* 189.
Warburg, O., and H. Christian, Biochem. Z. 1943. *314.* 149.
Ågren, G., Ark. Kemi, Mineral., Geol. 1942. *16 B.* No. 6.
Ågren, G., This journal 1945 a. In press.
Ågren, G., Ibidem 1945 b. In press.
Ågren, G., and J. Waldenström, Acta Med. Scand. 1944. *119.* 167.

From the Physiological Department, Karolinska Institutet, Stockholm.

Investigations into the Mechanism of the Liberation of Renin in Ischemic Kidneys.

By

JENS BING.

Received 14 November 1944.

Introduction.

When TIGERSTEDT and BERGMAN took up the question of the importance of the kidneys for the circulation, they were inspired by BROWN-SEQUARD's investigations into the internal secretion of the kidney. As we know, TIGERSTEDT and BERGMAN (1898) found in the cortex of the kidney a stuff which they called renin, the pressor effect of which they demonstrated in experiments on animals. They considered that the renal venous blood normally contained renin, and discussed the possibility that hypertension in man might be attributed to an abnormal liberation of renin from the kidneys into the blood.

The question as to the importance of renin for the pathogenesis of hypertension was brought into renewed prominence after the investigations of GOLDBLATT and his co-workers, in which it was shown that in laboratory animals, on partial occlusion of the renal arteries, a hypertension, which in later investigation was found to be due to an increased content of renin in the renal venous blood, could be produced.

Also after total ischemia, by complete occlusion of the renal vessels, a liberation of renin can be produced in dogs, cats and rats, as was first shown by DICKER (1937) and TAQUINI (1940) and afterwards, in extensive investigations, by PRINZMETAL, LEWIS and LEO (1940).

At first this giving-off of renin from the kidneys was cautiously mentioned. An abnormal "liberation" of renin was merely suggested as a possible cause of experimental hypertension in test animals and of renally induced hypertension in man. Latterly, however, it has become usual to talk of a "secretion" of renin. Thus PAGE and CORCORAN (1942) speak of an "increased secretion of renin" in animals with experimental renal hypertension. And BRAUN-MENENDEZ (1944) states that "whenever the blood pressure decreases, the normal kidney secretes renin, which through the formation of hypertensin will tend to restore normal blood pressure". Finally, it should be mentioned that in the "Annual Review of Biochemistry" renin during the last few years has been placed under the heading of hormones.

Content of Renin in Perfusates from Kidneys Perfused with Capillary-Injuring Substances.

In some previous investigations (BING 1944) it was confirmed that a pressor effect could be obtained in releasing a temporary total occlusion of the renal vessels in cats. And it was shown that a liberation of renin could be produced on total occlusion of the renal vessels and incubation of the kidneys in Ringer's solution. If rabbit kidneys are perfused immediately after extirpation, no renin (or scarcely any) will be found in the perfusate, whereas after incubation at about 2° some pressor substance, and after incubation at 37° considerably more, can be found, with a tendency to an increase in amount with the duration of the incubation. The liberated amount of renin will be quintupled to decupled if the kidneys before incubation are placed for about half an hour in 2 % phenol, toluol, ether or acetone.

It may *a priori* be regarded as scarcely probable that kidneys placed in Ringer's solution at 2° or at 37° would be able to maintain the capacity for secreting for many hours after extirpation. It is still more improbable that incubation in phenol, toluol, ether or acetone would be able to produce such a secretion. *It is far more probable that the mechanism in the liberation of renin is a diffusion through injured capillaries, the permeability of which has been increased. A similar increase of permeability may be expected to occur in the membrane of the renin-forming cells.*

If the increase in the renin content of the perfusate which is produced by placing the kidneys in phenol or toluol before incu-

bation is due to a change in capillary and cell permeability, it might be expected that an intra-vascular injection of these substances would produce a similar change. In order to ascertain whether this was the case, both kidneys were extirpated from a rabbit, one of them being perfused with 5 ml 2 % phenol, toluol or acetone, whilst the other, which served for control, was perfused with 5 ml Ringer's solution.[1] After being kept for 5—10 minutes at room temperature, each of the kidneys was perfused with as many ml Ringer (mixed with a little tricresol) as it weighed in grammes; the perfusion was repeated three times with the same amount of fluid. The renin content of the preparations was then examined after injection into a cat or dog narcotized with 5 cg chloralose per kg, and which had been pre-treated with 0.1 mg ergotamine tartrate and 1 mg atropine sulphate per kg of body-weight. Ergotamine was used because it had been shown by EULER and SCHMITERLÖW (1944) to have a suppressing action on the moderator nerve mechanism with a following smoothing effect on the blood pressure, as was often observed also during the present investigation.

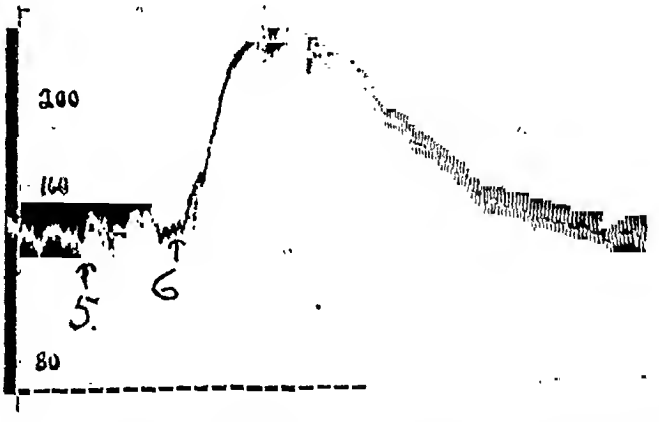


Fig. 1. Distinctly greater pressor effect of perfusate from a rabbit kidney pretreated with intravascular injection of phenol (Mark 6) than of perfusate from the other kidney from the same animal, which was pretreated with intravascular injection of Ringer's fluid (Mark 5).

As will be seen from Table I and Fig. 1, a distinct pressor effect was found on almost all occasions after the injection of perfusates from the kidney previously treated with phenol, toluol

[1] I am indebted to Professor WESTMAN, Karolinska Sjukhuset, for kindly supplying me with rabbit kidneys.

or acetone, whereas perfusates from the control kidneys merely produced slight rises in blood pressure. It is also shown in the table that a corresponding intravascular injection of a 10 per cent NaCl-solution gave similar results. An injection of saponin in the form of a 0.5 % *extractum fluidum senegae*, was also followed by a liberation of renin, which, however, was less marked.

Table 1.

Effect (in mm Hg) on the blood pressure of dogs or cats of perfusates from kidneys previously perfused with toluol, phenol, acetone, hypertonous NaCl solution or saponin, and from the corresponding control kidneys perfused with Ringer.

The bracketed tests were made with the same perfusate.

Test No.	Dosage in ml per kg	The kidney previously perfused with	Effect	Effect of control perfusate
104	0.50	Toluol	40	0
78	1.10	Toluol	32	10
82	0.50	Toluol	16	3
—	0.50	Toluol	16	3
—	0.50	Toluol	20	3
104	0.50	Phenol	90	10
—	0.25	Phenol	78	0
80	1.00	Acetone	86	12
—	0.25	Acetone	20	8
—	0.50	Acetone	36	4
—	0.25	Acetone	6	4
—	0.50	Acetone	10	5
—	0.50	Acetone	4	4
82	0.50	Acetone	20	4
80	1.00	10 % NaCl solution	20	6
—	1.00	10 % NaCl solution	20	12
—	0.50	0.5 % Extract. fluidum senegae	5	3
—	1.00	0.5 % Extract. fluidum senegae	10	10
—	1.00	0.5 % Extract. fluidum senegae	20	9
—	0.50	0.5 % Extract. fluidum senegae	9	4
—	1.00	0.5 % Extract. fluidum senegae	14	6

That the pressor substances occurring in the perfusate was renin was indicated by the configuration of the curve, by the thermolability of the substance, in that the effect disappeared on boiling, and by incubation tests with globulin, in which heat-stable hypertensin was formed.

Phosphatase Content in Renal Perfusates.

When it had been clearly indicated by the above investigations that the mechanism in the release of renin in incubated rabbit

kidneys must be a diffusion, it was of interest to investigate whether also other enzymes diffused out and could be shown in the perfusates. In order to ascertain this, phosphatase determinations were made with Buch and Buch's method (a modification of King and Armstrong's method), in which the samples are incubated with a solution containing phenylphosphoric acid, and the amount of phenol split-off is determined. With this method, according to BUCH, from 2 to 7 units of phosphatase are found in the serum of normal human beings. In the serum from some normal rabbits I have found similar values.

In perfusates produced in the above-mentioned manner, no phosphatase was found when the kidneys used were fresh, whereas with the use of incubated kidneys one found large concentrations of that substance, which increased with the duration of the incubation, as indicated in Fig. 2, which shows the values in perfusates from kidneys previously kept at room temperature in toluol for 30—40 minutes. In perfusates from kidneys which had been placed in Ringer's solution without previous toluol treatment, considerably lower values were found for phosphatase as well as for renin: for example after one hour 1.5, after three hours 6 and after five hours 30 units, that is about one-tenth of the value found in perfusates from kidneys pre-treated with toluol.

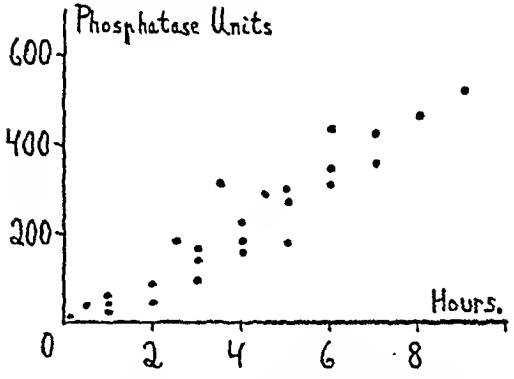


Fig. 2. The figure shows the relation between the phosphatase content of perfusates of rabbit kidneys and the incubation time in Ringer's fluid at 37° after pretreatment with toluol.

For a more detailed comparison between the contents of renin and phosphatase in renal perfusates, it was found expedient to determine the renin concentration after conversion into hypertensin.

This was done by incubating a suitable amount of perfusate in 15 ml Ringer with 20 ml globulin solution for 10 minutes, after which the mixture was boiled and the precipitate centrifuged off, washed and centrifuged again. The mixtured centrifugates were filtered and concentrated by evaporation in a vacuum to 5 ml. The hypertensin solution thus produced was compared

in the blood-pressure tests on cats with a standard hypertensin solution produced in such a way that the pressor effect of 0.1 ml corresponded to the effect of 0.1 mg. tyramin (EDMAN, EULER, JORPES and SJÖSTRAND (1942)[1].

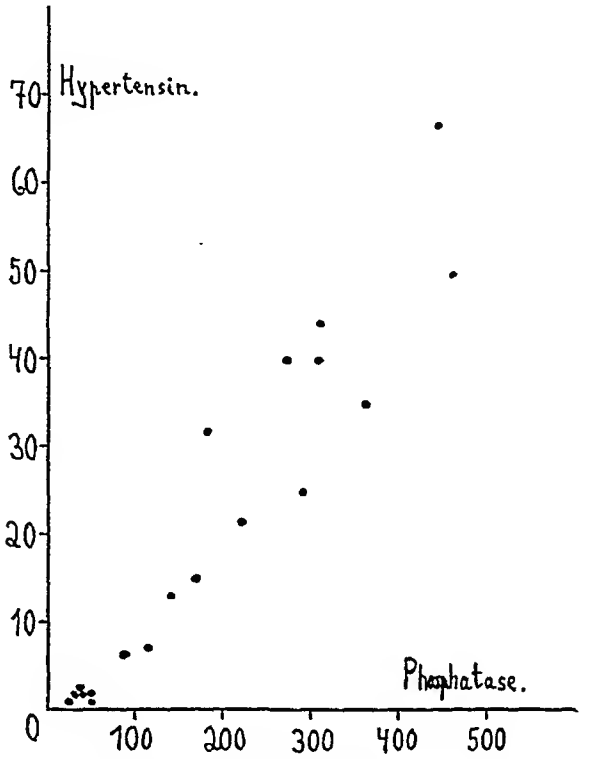


Fig. 3. The figure shows the relation between hypertensin and phosphatase content of perfusates of incubated rabbit kidneys.

If with 0.1 ml of the unknown hypertensin preparation (x hypertensin) one obtains the same effect as with 0.1 ml standard hypertensin, it is considered that there are 200 renin units in the perfusate, provided that 0.1 ml of the perfusate has been used for the globulin incubation. The unit is accordingly computed by dividing 200 by 10 times the number of cc in the perfusate, multiplied by $\frac{\text{x hypertensin}}{\text{standard hypertensin}}$.

As will be seen from Fig. 3, there is a distinct relation between the phosphatase and the renin content of the perfusate.

[1] My thanks are due to Dr. EDMAN for kindly supplying me with such a standard solution and with globulin. I am greatly indebted to Docent B. JOSEPHSON for kind permission to make the determinations of phosphatase in the medico-chemical laboratory, Karolinska Sjukhuset.

Comments.

The close relation between the content of renin and phosphatase in renal perfusates, like the above-mentioned perfusion tests, indicates that the mechanism in the liberation of renin in the totally ischemic kidneys is a diffusion through injured capillarics and cell membranes, the permeability of which has become abnormally marked.

These experiments, of course, tell us nothing about the mechanism in the liberation of renin from the partially ischemic kidneys found in animals with experimental hypertension and in the kidneys of hypertonia patients. But we must reckon with the possibility that we are concerned here too not with an abnormally large secretion, but with a lesion of the capillaries and kidney cells accompanied by an abnormal discharge of renin. Hypertonia in fact occurs in a number of renal lesions in which the capillaries and cells are known to be injured. And from lesions of other parenchymatous organs it is known that the enzymes can diffuse out into the blood, just as is the case with the quinine-resisting lipases in liver lesions and with diastasis in lesions of the pancreas.

Summary.

The mechanism for the liberation of renin in totally ischemic kidneys was ascertained in investigations showing that, by perfusing the kidneys with various capillary-injuring substances such as toluol, phenol, acetone, hypertonous salt solution and saponin, one obtains a liberation of renin which is best explained as a diffusion through injured capillaries and cell membranes, the permeability of which has been increased. This view is also borne out by examining the content in the perfusates of another renal enzyme, namely phosphatase. There is in fact a rather close relation between the content of renin and phosphatase. We must reckon with the possibility that the same mechanism is found in the abnormal liberation of renin in renally induced hypertension in man and animals.

The present investigation was supported by a grant from the *Nobel Foundation.*

References.

Bing, J., Acta physiol. scand. 1944. *8.* 348.

Braun-Menendez, E., Ann. Rev. Physiol. 1944. *6.* 265.

Buch, I. and H. Buch, Acta med. scand. 1939. *101.* 211.

Dicker, E., C. R. Soc. biol., Paris 1937. *126.* 88.

Edman, P., U. S. v. Euler, E. Jorpes and V. T. Sjöstrand, J. Physiol. 1942. *101.* 284.

Euler, U. S. v. and C. G. Schmiterlöw, Acta physiol. scand. 1944. *8.* 122.

Leo, S. D., M. Prinzmetal and H. A. Lewis, Amer. J. Physiol. 1940—41. *131.* 18.

Lewis, H. A., S. D. Leo, and M. Prinzmetal, Heart 1941. *21.* 319.

Page, I. H. and A. C. Corcoran, Advances Intern. Med. 1942. *1.* 135.

Prinzmetal, M., H. A. Lewis, and S. D. Leo, Proc. Soc. exp. Biol., N. Y., 1940. *43,* 696.

Prinzmetal, M., H. A. Lewis, J. Taggart, H. Wilkins, and D. R. Drury, Heart 1940. *20.* 525.

Prinzmetal, M., H. A. Lewis, and S. D. Leo, J. exp. Med. 1940. *72.* 763.

Taquini, A. C., Heart 1940. *19.* 513.

Tigerstedt, R., and P. G. Bergman, Skand. Arch. Physiol. 1898. *8.* 223.

From the Department of Physiology, University of Lund, and the Gynaecological Clinic, Karolinska Sjukhuset, Stockholm.

On the Structure and Function of the Mammary Glands after Hypophysectomy and Transection of the Hypophyseal Stalk in Rats.

By

D. JACOBSOHN and A. WESTMAN.

Received 2 January 1945.

Since the discovery of the lactogenic hormone by STRICKER and GRUETER (1928) numerous investigators have shown that the anterior lobe of the pituitary exercises a direct control over milk secretion (lit.: NELSON, 1936, TURNER, 1939, FOLLEY, 1940, RIDDLE, 1940). No satisfactory explanation has as yet been reached, however, either of the mechanism by which the anterior lobe hormones necessary for lactation are released, or of the question as to what extent the secretory activity of the mammary gland cells is dependant on other than hormonal factors.

HAMMOND (1936) has collected evidence from experiments of his own and of others which support his theory that the relief of pressure on the secreting cells by the withdrawal of milk is of fundamental importance for the secretory process. A reflex released by the tactile stimulus at the nipple, resulting in a contraction of smooth muscles and a stasis of circulation in the mammary gland, brings about the "letting down" of milk.

SELYE (1934) on the other hand suggests that the suckling stimulus excites a reflex, in which afferent impulses cause the discharge of lactogenic anterior pituitary hormones. SELYE's assumption of a neuro-hormonal reflex mechanism is supported by the experiments of SELYE, COLLIP and THOMSON (1934), INGELBRECHT (1935), and HOOKER and WILLIAMS (1939).

HEROLD (1939) and DESCLINS (1940) found in rats that transection of the pituitary stalk during lactation caused cessation of

milk secretion and death of the young. This finding indicates that the reflectory impulses, as postulated by SELYE, reach the anterior pituitary body by way of the hypothalamus and the pituitary stalk. Separation of the nervous connections between the hypophysis and the midbrain prevents the discharge of the anterior lobe hormones, necessary for lactation.

If this is true, the structural changes occurring in active mammary glands after transection of the pituitary stalk should be similar to those developing after hypophysectomy.

HEROLD and DESCLINS did not, however, follow the mammary gland changes microscopically, nor did they compare these changes with those occurring at corresponding stages after hypophysectomy or after weaning the young from normal lactating rats.

Feeling the need for comparative experiments of this kind, we studied the mammary gland changes developing after weaning the young from normal lactating rats, after hypophysectomy or after transection of the hypophyseal stalk.

Methods.

The present investigation was carried out on pregnant and lactating albino rats, which had either been deprived of their young, hypophysectomized or had the pituitary stalk cut.

The pituitary stalk is reached by the parapharyngeal route and severed outside the connective tissue capsule which surrounds the hypophysis. The bone lamina of the basisphenoid underlying the pituitary body remains unopened, thus avoiding surgical lesions of the hypophysis. — Other investigators who performed stalk transections on rats by the parapharyngeal route (e. g. RICHTER, 1933) have exposed the anterior pole of the hypophysis, separating the latter from the stalk inside the dural capsule.

Apart from the procedure given below the operation is performed in the same way as a hypophysectomy (SELYE, COLLIP and THOMPSON, 1933). The posterior belly of the digastric muscles is divided. Without entering the buccal or nasal cavities the sphenoid bone is approached and freed rostrally until the intertransversal sinus, which appears as a dark transverse line, is clearly visible. The sinus is opened with a dental drill, and for a short time a small paraffin ball is pressed against the drill-hole in order to stop bleeding. The drill-hole is enlarged and the dura mater split with a fine surgical needle. The pituitary stalk is now exposed and severed by means of a fine pointless hook just broad enough to carry the hypophyseal stalk.

At the end of the experiment we controlled microscopically whether the pituitary stalk had been completely cut. The microscopic examination was performed on haematoxylin-eosin stained serial sections

through the hypothalamus with the hypophysis attached. In 3 out of 17 cases the control revealed a small remnant of the stalk. These 3 animals were discarded.

When the pituitary stalk is transected according to the technique mentioned above the hypophysis shows the same structural changes as those described by WESTMAN, JACOBSOHN and HILLARP (1933) when using another method.

As a rough indication of the mammary gland activity the stomach contents and body-weight curves of the young were controlled. This was, however, not always possible. When the starving sucklings became too weak, they were exchanged with litters from normal lactating rats in order to supply the mammary glands with a constant suckling stimulus. In this way, in series 2—5 all animals were supplied with healthy litters until the experiment was concluded. The mammary glands were examined microscopically at different stages of the experiment.

The glands were extirpated and fixed in a 10 per cent formalin solution, embedded in paraffin, cut at 5 μ and stained with haematoxylin-eosin. Peripheral and central sections of the gland were examined.

As the changes in the mammary glands occurring in the different experimental series appeared sufficiently distinct in this way, we refrained from using more elaborate cytological methods.

Material.

The material for this investigation consists of 82 mammary glands from 25 albino rats after

1) weaning the young after a period of normal lactation (table 1),
2) hypophysectomy during the second half of pregnancy (table 2),
3) hypophysectomy during established lactation (table 3),
4) transection of the hypophyseal stalk during the second half of pregnancy (table 4),
5) transection of the hypophyseal stalk during established lactation (table 5).

The identification number for each rat is given in the second column of the tables, thus making it possible to follow the changes, developing in the mammary glands from one animal. — The amount of the parenchyma and stroma is marked by cross signs, "parenchyma (p) ++++, stroma (str.) 0" corresponding to the gland volume during normal lactation, "p. +. str. ++++" corresponding to an inactive gland of an adult female rat.

Results.

As far as we know no systematic investigations of the mammary gland changes following transection of the pituitary stalk have as yet been performed. For comparative purposes, the structural

changes occurring in normal and hypophysectomized animals are described in paragraph 1—3. They agree in essentials with those of earlier investigators (lit. TURNER, 1939).

1) *Mammary gland changes after weaning the young during normal lactation* (c. f. MAEDER, 1922).

Table 1.

Mammary gland changes after weaning the young during normal lactation.

Exp.	Rat no.	Mammary gland days after		Microscopic examination
		parturition	weaning	
1	4	1	1	p: ++++ str: 0
2	5	9	2	Widely distended acini, empty or filled with secretion. Acinar cells vacuolated and with uneven indistinct boundaries on the side of the lumen and large vesicular nuclei.
3	4	3	3	p: +++ str: ++
4	1	5	3	Acini distended with secretion. Lumina smaller than in exp. 1. A few collapsed acini. A number of acinar cells as before, most of them however with one large vacuol and irregularly outlined deeply stained nuclei.
5	2	8	4	p: ++ str: ++
6	3	9	4	Acini collapsed, but a few still with a small lumen distended with secretion. Ducts well distended with secretion.
7	4	5	5	p: + str: +++
8	2	10	6	Acini as before. Number of acinar cells,
9	3	11	6	however, reduced. Only a few ducts contain
10	5	13	6	secretion within the lumen.
11	4	7	7	
12	2	12	8	p: + str: ++++
13	3	13	8	Acini and most of the smaller ducts collapsed. Number of acinar cells further reduced. Only a few small ducts contain secretion within the lumen.
14	2	14	10	
15	3	15	10	
16	5	17	10	
17	5	20	13	

Table 1 contains 17 experiments performed on 5 normally lactating rats, from which the litters had been weaned 1—7 days after parturition. 1—13 days after weaning, we extirpated the mammary glands and examined them microscopically.

On the 1st and 2nd day after weaning the mammary gland is still lactating. On the 3rd day (exp. 3 and 4) the parenchyma begins to be reduced in proportion to the stroma. Most of the acinar cells show

signs of degeneration and ceasing secretory activity. The parenchyma of glands studied at the 4th—7th day stages (exp. 5—11) is steadily decreasing. On the 8th day the gland has reached a more advanced stage of involution. The microscopic examination reveals an inactive gland, and this picture shows practically no change until the last day on which the glands were studied, that is the 13th day after weaning.

Lactation ceases about 3 days after weaning the young from normally lactating rats. In about 8 days after weaning the mammary gland reaches an advanced stage of involution.

2) *Rats hypophysectomized during the second half of pregnancy* (c. f. JEFFERS, 1935).

Table 2.

Mammary gland changes occurring after hypophysectomy during the second half of pregnancy.

Exp.	Rat no.	Mammary gland days after		Microscopic examination
		operation	parturition	
1	414	1	0	p: ++++ str: 0 Widely distended acini, most of them contain secretion within the lumen. Acinar cells with large vacuoles and large vesicular nuclei. Cell boundaries on the side of the lumen uneven and indistinct. In some places irregularly outlined deeply stained nuclei.
2	413	2	0	p: ++ str: ++ As exp. 1, acini, however, with a very small lumen.
3	388	5	0	As exp. 1, acini, however, less distended.
4	414	2	1	p: +++ str: ++
5	394	6	1	As exp. 1, acini, however, less distended and
6	395	7	1	most of the nuclei irregularly outlined and deeply stained.
7	414	3	2	p: +++ str: + As exp. 4—6. Some acini collapsed. Nuclei irregularly outlined and deeply stained.
8	413	4	2	p: ++ str: +++
9	394	7	2	Most of the acini collapsed. Ducts distended with secretion.
10	413	5	3	p: ++ str: ++++ Acini and small ducts collapsed. Number of acinar cells reduced.
11	388	8	3	p: ++ str: +++
12	395	9	3	Acini collapsed. Very little secretion left
13	396	10	3	within the lumen of the ducts.

Exp.	Rat no.	Mammary gland days after		Microscopic examination
		operation	parturition	
14	414	5	4	p: ++ str: +++
15	394	9	4	Acini and smaller ducts collapsed. Acinar
16	396	11	4	cells disappearing.
17	413	7	5	p: ++ str: ++++
18	395	11	5	Number of parenchymal cells more reduced
19	396	12	5	than before.
20	394	11	6	Further reduction of gland parenchyma until
21	388	12	7	p: + str: ++++. Small islets of parenchy-
22	414	11	9	mal cells within a stroma of adipose and
23	395	15	9	connective tissue.
24	396	16	9	
25	413	11	10	

Table 2 shows 25 experiments on 6 rats, hypophysectomized 1—7 days before parturition. 0—10 days after parturition (1—16 days after operation) the mammary glands were excised and studied microscopically.

On the day of parturition the mammary glands of exp. 1 and 3 (1 and 5 days after operation) are well developed and there was secretion but not so abundant as in normal glands. The gland of exp. 2 (2 days after operation) shows only slightly distended acini, however, and the parenchyma is diminished in proportion to the stroma. 1 day after parturition (2, 6, 7 days after operation) many of the acinar cells seem to discharge secretion, while most of them are degenerating. On the 2nd day after parturition (exp. 7—9) lactation has ceased. The parenchyma of the glands examined 3—5 days after parturition (exp. 10—19) is rapidly diminishing, and 6—10 days after parturition (exp. 20—25) a complete involution is found.

After parturition the mammary glands of rats, hypophysectomized during the second half of pregnancy, undergo a short lactation period *(lasting about 1 day) followed by a rapid involution* of the entire parenchyma in the course of 3—6 days.

The involution occurring in the mammary glands of rats, hypophysectomized during lactation corresponds to that observed in rats hypophysectomized during the second half of pregnancy.

3) *Rats hypophysectomized during established lactation.*

Table 3.

Mammary gland changes occurring after hypophysectomy during established lactation.

Exp.	Rat no.	Mammary gland days after		Microscopic examination
		parturition	operation	
1	383	6	1	p: ++++ str: 0 Acini widely distended with secretion. Acinar cells vacuolated and with uneven indistinct boundaries on the side of the lumen. Large vesicular nuclei. In some places acinar cells with one large vacuol and irregularly outlined deeply stained nuclei.
2	385	8	2	p: +++ str: ++ Acini with a small lumen or collapsed. Ducts and some acini distended with secretion. Acinar cells: very few vacuolated and with large vesicular nuclei, most of them with one large vacuol and irregularly outlined deeply stained nuclei.
3	381	12	2	p: +++ str: + Acini medium-sized, some collapsed. Otherwise as in exp. 2.
4	386	11	3	p: ++ str: ++ Many collapsed acini. Ducts and some acini distended with secretion. Acinar cells with irregularly outlined deeply stained nuclei. Many acinar cells with one large vacuol.
5 6	384 381	9 15	4 4	p: ++ str: +++ Acini collapsed. Very little secretion left within the lumen of the small ducts. (In exp. 6 in some places medium-sized acini distended with secretion.)
7 8	384 386	12 15	7 7	p: + str: +++ Groups of acinar cells around ducts. No secretion left in the lumina of the ducts.
9 10	386 381	17 20	9 9	p: + str: ++++ As before.
11 12 13	384 386 384	16 20 18	11 12 13	As before. Further progressing involution.

Table 3 shows 13 experiments on 5 rats, hypophysectomized during the lactation period. The mammary glands were removed 1—13 days after hypophysectomy.

During the first 2 days after hypophysectomy (exp. 1—3) secreting cells are present. On the 3rd day after the operation (exp. 4) the acinar cells degenerate and on the 4th day the entire gland parenchyma is reduced. During the following days the involution progresses rapidly.

4) *Rats with the hypophyseal stalk transected during the second half of pregnancy.*

Table 4.

Mammary gland changes occurring after stalk transection during the second half of pregnancy.

Exp.	Rat no.	Mammary gland days after operation	Mammary gland days after parturition	Microscopic examination
1	298	3	0	p: +++ str: ++
2	288	6	0	Widely distended acini, empty or filled with
3	297	5	1	secretion. Acinar cells vacuolated and with
4	290	8	1	irregular uneven boundaries on the side of
5	288	7	1	the lumen. Large vesicular nuclei. In large
6	268	8	2	areas, however, acinar epithelium with one large vacuol and irregularly outlined deeply stained nuclei.
7	297	7	3	p: +++ str: ++
8	288	9	3	As before. Acini less distended.
9	298	7	4	p: +++ str: +++ Acini in some areas as before, in others small or collapsed. Acinar epithelium of the larger acini as in exp. 1—6.
10	297	9	5	p: +++ str: +++ As exp. 9, but more acini widely distended with secretion.
11	290	13	5	p: ++ str: +++ Acini small, most of them collapsed. In some places larger acini. These and the ducts distended with secretion. The acini with a larger lumen had an epithelium as in exp. 1—6.
12	290	14	6	p: + str: +++ As before. The lumina of ducts and alveoli contain only little secretion.
13	298	10	7	p: ++ str: +++ As before.

Table 4 shows 13 experiments on 4 rats, with the hypophysis stalk transected 3—7 days before parturition. Mammary glands, removed 0—7 days after parturition (3—14 days after operation) were examined microscopically.

Until the 2nd day after parturition most of the acinar cells are secreting. The parenchyma is diminished in proportion to the stroma. 3 days after parturition (exp. 7 and 8) most of the cells are secreting. The secretion is, however, less abundant than before. On the 4th day after parturition (exp. 9) milk secretion is observed in same areas but most of the glandular tissue is degenerating. One of the glands, excised on the 5th day after parturition (exp. 10) corresponds to the gland removed on the day before, while the other (exp. 11) shows a more advanced stage of involution. Despite the involutionary changes, lactating acini are still present. The following two experiments (12 and 13) are similar to exp. 11. Here, too, the glands, removed 6 and 7 days after parturition respectively, show an advanced stage of involution. The parenchyma, however, contains some lactating acini.

In contrast to the total involution occurring after hypophysectomy, the involution of the mammary gland in rats with the pituitary stalk transected before parturition does not affect the entire gland. A few lactating acini can still be found, while most of the glandular tissue has disappeared.

5) *Rats with the hypophyseal stalk transected during established lactation.*

Table 5.

Mammary gland changes occurring after stalk transection during established lactation.

Exp.	Rat no.	Mammary gland days after		Microscopic examination
		parturition	operation	
1	299	9	1	p: +++ str: 0 Widely distended acini. Lumina empty or filled with secretion. Acinar cells vacuolated and with uneven indistinct boundaries on the side of the lumen. Large vesicular nuclei. (Exp. 4: a great number of acinar cells with ono large vacuol.)
2	300	9	1	
3	301	7	2	
4	299	10	2	
5	292	10	3	p: +++ str: + As exp. 4. Acini smaller, a few of them collapsed.
6	292	11	4	p: ++ str: +++ Collapsed and up to medium-sized acini. Acini and ducts distended with secretion. Epithelium with one large vacuol and irregularly outlined deeply stained nuclei. In some areas acinar cells as in exp. 1—3.

Exp.	Rat no.	Mammary gland days after		Microscopic examination
		parturition	operation	
7	301	10	5	p: ++ str: +++ Small or collapsed acini. Little secretion left within the lumina of the ducts. In some areas larger acini distended with secretion; their epithelium as in exp. 1—4.
8	299	14	6	p: +++ str: ++ As exp. 6.
9	284	10	7	p: ++ str: +++ Acini small or collapsed. Little secretion left within the lumina of the acini and ducts. Acinar cells with irregularly shaped deeply stained nuclei. Some acinar cells with one large vacuol.
10	300	15	7	As exp. 7.
11	301	13	8	Large area showing the same picture as exp. 1—3. In other places greatly reduced glandular tissue.
12	299	16	8	As exp. 6.
13	300	17	9	As exp. 11.
14	284	14	11	p: ++ str: +++ As exp. 9, but with a few vacuolated acinar cells with large vesicular nuclei.

Table 5 shows 14 experiments on 5 lactating rats, on which stalk transection was performed 3—8 days after parturition. The mammary glands were extirpated 1—11 days after the operation.

For 2 days after the operation (exp. 1—4) microscopic examination reveals a normally lactating gland. One experiment, however, (exp. 4) shows many degenerating acinar cells. On the 3rd day (exp. 5) the parenchyma is somewhat reduced in proportion to the stroma. Otherwise the gland is similar to that of exp. 4. On the 4th day (exp. 6) the acini begin to disappear and the stroma increases. In some parts, however, normal lactation is still present. On the 5th day (exp. 7) the involutionary changes are more advanced, but here, too, secreting cells can be observed. The gland removed on the 6th day (exp. 8) is similar to that of the 4th day. On the 7th day, exp. 9 shows involution and contains no secreting cells. Exp. 10 (also from the 7th day) corresponds, however, to exp. 7, where some parts of the gland are still lactating. 8 days after the operation, exp. 11 shows a large region with acini fully resembling those of normal lactating glands. In other places an advanced stage of involution has been reached. In the following three experiments, 12, 13, 14 (8, 9, 11 days after operation), the glandular tissue is also on the whole greatly reduced, but a few alveoli, distended with secretion and with secreting cells, are still present.

The mammary glands of rats with the pituitary stalk transected during lactation show mainly the same changes as those found in rats on which stalk transection was performed during the second half of pregnancy. While most of the parenchyma undergoes a more or less rapid involution, milk secretion still goes on in some parts of the gland.

Discussion.

Our results confirm the observation of HEROLD (1939) and DESCLINS (1940) that the sucklings of rats which have undergone stalk transection die from starvation. The present experiments show, however, that *the mammary gland involution occurring after stalk transection differs markedly from the total involution, occurring after hypophysectomy or after weaning the young from normal lactating rats. After transection of the hypophyseal stalk milk secretion still continues in some parts of the gland.*

As there are considerable differences in the reaction of the hypophysectomized and the stalk-transected rats, experiments on the latter are unsuitable as a support of the assumption (HEROLD, 1939, DESCLINS, 1940) that the discharge of lactogenic anterior lobe hormones is controlled by the diencephalon. — In interpreting the mammary gland changes occurring in stalk transected rats the following points must be considered:

1) Transection of the hypophyseal stalk cannot be performed without destroying the hypophyseal portal system. The circulatory disturbances occurring in the pituitary body do not disappear immediately (WESTMAN, JACOBSOHN and HILLARP, 1943). During this time production of anterior lobe hormones may be inhibited. 2) In the rat the mammary gland involution occurring after hypophysectomy or after weaning is very rapid. — When the anterior pituitary lobe again begins to discharge a sufficient amount of lactogenic hormones, the involution may have progressed so far that most of the parenchyma is refractory to the hormonal stimuli.

Thus, the experiments on stalk transected rats do not furnish support for SELYE's theory, assuming that lactation is controlled by a neuro-hormonal mechanism. The investigation of other species with a protracted involution period may help to elucidate this problem. Experiments on stalk-transected rabbits will be dealt with in a further publication.

Summary.

1) A technique of transecting the hypophyseal stalk in rats parapharyngeally, outside the dural capsule and without exposing the pituitary body is described.

2) The mammary gland changes in rats occurring after weaning, after hypophysectomy and after stalk transection are studied microscopically.

After weaning and after hypophysectomy a total involution of the mammary gland occurs. After stalk transection there is an involution, which, however, does not affect the entire gland. Some lactating acini can still be found when most of the glandular tissue has disappeared.

References.

Collip, J. B., H. Selye and D. L. Thompson, Virchows Arch. 1933. *290*. 24.
Desclins, L., R. Soc. Biol. Paris 1940. *134*. 267.
Folley, S. J., Biol. Rev. 1940. *15*. 421.
Hammond, J., Vet. Rec. 1936. *16*. 519.
Herold, L., Arch. Gynäk., 1939. *168*. 534.
Hooker, Ch. W. and W. L. Williams, Yale J. Biol. a. Med. 1939. *12*. 559 (quoted from Ann. Rev. Physiol. 1941. *3*. 605).
Ingelbrecht, P., R. Soc. Biol. Paris 1935. *120*. 1369.
Jeffers, Katherine R., Amer. J. Anat. 1935. *56*. 279.
Maeder, L. M. A., Amer. J. Anat. 1922. *31*. 1.
Nelson, W. O., Physiol. Rev. 1936. *16*. 488.
Richter, C. P., Amer. J. Physiol. 1933. *106*. 80.
Riddle, O., J. Amer. med. Ass. 1940. *115*. 2276.
Selye, H., Amer. J. Physiol. 1934. *107*. 535.
Selye, H., J. B. Collip and D. L. Thompson, Endocrinology 1934. *18*. 237.
Turner, C. W., "The Mammary Glands" Sex and Internal Secretions. London 1939.
Westman, A., D. Jacobsohn and N.-Å. Hillarp, Mschr. Geb. u. Gynäk. 1943. *116*. 225.

From the Department of Physiology, University of Lund.

Further Attempts to Isolate a Gastric Secretory Excitant from the Pyloric Mucosa of Pigs.

By

BÖRJE UVNÄS.

Received 12 January 1945.

In a previous paper MUNCH-PETERSEN, RÖNNOW and UVNÄS (1944) presented the results of their investigations on the physiological and chemical properties of a gastric secretory principle from the pyloric mucosa of pigs. In this and other papers from this laboratory (UVNÄS 1942, 1943, BAUER and UVNÄS 1944) the previous works in this field as also the problems on the possible rôle of a pyloric principle in the humoral regulation of the cephalic and chemical phases of the gastric secretion were discussed. A repeated review of the extensive litterature is therefore omitted here.

The secretory principle isolated by MUNCH-PETERSEN, RÖNNOW and UVNÄS is a protein-like substance. It stimulates the HCl-glands of the gastric mucosa but shows no other histamine-like properties. It was obtained from HCl-extracts of the pyloric mucosa by precipitation with 20 % NaCl and reprecipitation with tannic acid or trichloracetic acid. After removal of inert material by washing with 80 % acid alcohol and isoelectrical precipitation at pH 8 the active material was finally precipitated with trichloracetic acid. The procedures used yielded highly active preparations. Due to the great consumption of trichloracetic acid the methods were too expensive for routine use, however. The trichloracetic acid fetched a high price and due to the war it was available only in limited quantities. To get an active material for further studies on the physiological and chemical properties of the secretory principle other preparative procedures had to be tried.

Experimental.

The experiments were performed on chloralose-urethane narcotized cats (0.02 g chloralose and 0.5 g urethane per kg body weight), previously starved for 24 hours. The trachea was cannulated and the oesophagus ligated. The stomach was drained by a thin perforated rubber tube introduced through a glass cannula inserted in the stomach wall just proximal to the pyloric region. The cannula with the rubber tube was pushed out through a stab wound in the left abdomen. The duodenum was ligated proximal to Vater's ampulla. After the abdominal wall had been closed the animal was placed on its left side, the front half somewhat higher to facilitate the outflow of the gastric juice. Then the animal was left for at least an hour before the testing was started.

The material to be tested was dissolved in physiological saline at about 60° C., and the pH of the solution brought to about 7.4 by adding a few drops of N/10 NaOH. The solution was injected into the iliac vein at a rate of 0.4 ml per minute. Usually the injection was stopped after 20 minutes and the secretion was then allowed to decline to the basic level. A new test was not made until 60 minutes had elapsed.

The secretory volume was measured every 15 minutes. The secretion during 60 minutes following the beginning of an injection was taken as a measure of the activity of the extracts. In order to facilitate the comparison of the activity of different preparations this was expressed in secretory units. The quantity of material causing the secretion of 1 ml strongly acid gastric juice in a cat weighing 2—3 kg was taken as containing a secretory unit. If possible doses initiating a secretion of 5—15 ml in 60 minutes were chosen. A secretory response below 2—3 ml was taken to be inconclusive.

It was previously observed that in some instances the secretory response to the preparations declined during the course of an experiment. The material was therefore tested for its activity repeatedly and on different cats.

The total acidity of the gastric juice was determined colorimetrically, phenolphtalein being used as indicator and N/10 NaOH as base.

The peptic activity of the gastric juice was determined by a modification of the Anson and Mirsky hemoglobin method. This will be described later in this paper.

Preparation of the Secretory Principle.

1. *Extraction with HCl.* The pyloric portions of stomachs from recently killed pigs were kept on ice and carried from the slaughter-house to the laboratory within 1—2 hours. The stomachs were washed under running tap-water and the mucosa removed. It was ground in a mincing-machine and then thrown down into boiling N/10 HCl. 200 ml HCl were used per stomach. After boiling for 15—20 minutes the material was left at room temperature over night.

II. *Precipitation of inert material at pH 3—4.* The following day the mucosal fragments were removed by filtering through gauze. By adding N NaOH the mixture was brought to an acidity of pH 3—4. A precipitate containing inert material was removed by centrifuging for 10 minutes. Care must be taken not to exceed pH 4, active material then being removed with the inactive. The centrifugate was filtered through cotton wool to remove fatty substances floating on the fluid.

III. *Precipitation with NaCl.* Sodium chloride was dissolved in the filtrate to give a concentration of 10 %. By adding the same volume of saturated sodium chloride solution a concentration of about 20 per cent was obtained. A precipitate formed. After 1—2 hours the material was centrifugated at high speed for about 10 minutes. The supernatant fluid was removed and the sediment collected in a glass vessel. To remove as much water as possible the precipitate was centrifuged once more for 30 minutes. It was then washed several times with acetone in great excess. During this procedure the precipitate acquired a tenacious consistency and stuck to the vessel walls. The washing had to be repeated until this consistency had quite disappeared and the material had become quite dry and crackled like sand against the vessel walls.

IV. *Removal of inert material by dissolving in N/10 HCl at 60° C.* The dried NaCl-precipitate was suspended in N/10 HCl, 700 ml being used for a quantity of dry material corresponding to 15 stomachs. During frequent stirring for 30 minutes at 60° C. considerable amounts of inactive material went into solution. The active material remained undissolved. It was separated from the solution by centrifugation and dissolved in 1,000 ml N/10 HCl at 100°. The solution was cooled to room temperature and was then brought to an acidity of pH 3—4 by adding N NaOH. A precipitate formed containing inert material, which was removed by centrifugation.

V. *Precipitation with tannic acid.* 5 ml of a 5 per cent tannic acid solution per stomach were added to the centrifugate. A heavy precipitate appeared, which was separated by centrifugation during five minutes. The precipitate was very difficult to get dry in acetone. The washing had to be repeated numerous times. The material was finally washed twice in ether.

VI. *Precipitation of inert material at pH 8.* The dried tannic acid precipitate was dissolved in a 0.9 % solution of NaCl at 40° C. 1,000 ml saline were used for an amount of dry material corresponding to 15 stomachs. The saline had to be added successively under frequent stirring as otherwise the material did not go into solution. N NaOH was added until a pH of 8 was reached. A precipitate formed, which after about an hour was removed by centrifuging.

VII. *Isoelectrical precipitation of active material.* To the centrifugate obtained as described above N HCl was added. Between a pH of 4.0—5.5 a precipitate formed containing active material. After adjusting the pH to the desired value the acid solution was allowed to stand at a temperature of 8—10° C. for 24 hours. The precipitate was then centrifuged off, washed several times in acetone, twice in ether, and dried in air. A complete flocculation of the active material was ob-

tained at a pH of about 4.4. A more selective precipitation was obtained at a pH of about 5.0, the preparations being more active. At this pH, however, a loss of active material up to 50 per cent occurred. By adding small amounts of $CuSO_4$, 1—2 mg per 100 ml, most of the remaining active material could be precipitated.

The Activity of the Preparations.

By isoelectrical precipitation at pH 4.8—5.2 a material of surprisingly high activity was obtained. Our most active preparations showed an activity of one secretory unit per 0.1 mg dry substance. Usually the activity varied between one secretory unit per 0.1—1 mg of dry material. The precipitate obtained by precipitation in the presence of $CuSO_4$ usually amounted to one secretory unit per mg. In view of the limited facilities at our disposal we have desisted from a further purification of the preparations.

By continuous intravenous injection of histamine it is possible to initiate and maintain in cats a steady secretion of strongly acid gastric juice. According to our experience an administration of 10—15 γ histamine biphosphate per kg body weight and minute is necessary to evoke a gastric secretion of 5—10 ml per 15 minutes. As seen from the experiment referred to in fig. 1 the intravenous injection of a pyloric preparation for 15 minutes at a concentration of about 20 γ per kg body weight and minute causes a considerable gastric secretion.

The Distribution of the Gastric Secretory Principle.

In a previous communication (Uvnäs 1943) it was reported that the secretory principle was predominantly localized at the pyloric mucosa. In those experiments we worked on crude preparations only precipitated from HCl-mucosal extracts by trichloracetic acid. As the presence of a secretory principle in such an impure material may be masked by the interference of inhibitory substances, investigations on the localization of the secretory principle were repeated. Fundic and duodenal mucosa, liver, spleen and skeletal muscle were investigated for their content of a gastric secretory principle. No active material was obtained. To be sure of a maximal yield of active material in these experiments the process IV from the scheme above was excluded and the isoelectrical precipitation performed in the presence of $CuSO_4$.

The Secretory Response to the Pyloric Principle.

MUNCH-PETERSEN, RÖNNOW and UVNÄS have previously described the secretory response to purified substances as regards the volume and acidity of the secretion. The secretory response to our preparations did not differ from their findings. A typical secretory curve is seen in fig. 1. These authors reported that the secretory response to their purified substances sometimes declined during the course of an experiment. Unfortunately this was also the case when our isolectrically precipitated preparations were used. Some of the preparations, which in themselves were highly active, considerably inhibited the excitatory effect of the same or of another active material. In some experiments the inhibition persisted throughout the experiment, in others the secretory responsibility reappeared in 3—4 hours. This is seen in fig. 2. The injection of a pyloric substance evoked the

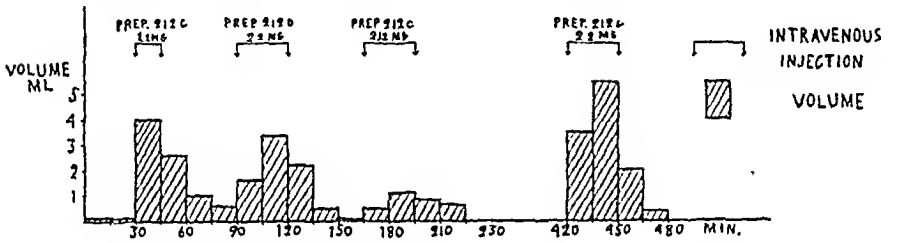


Fig. 1. Cat 3.3 kg. Gastric secretion after slow intravenous injection of two different pyloric preparations. Note the decline of the secretory response and the augmented response four hours later.

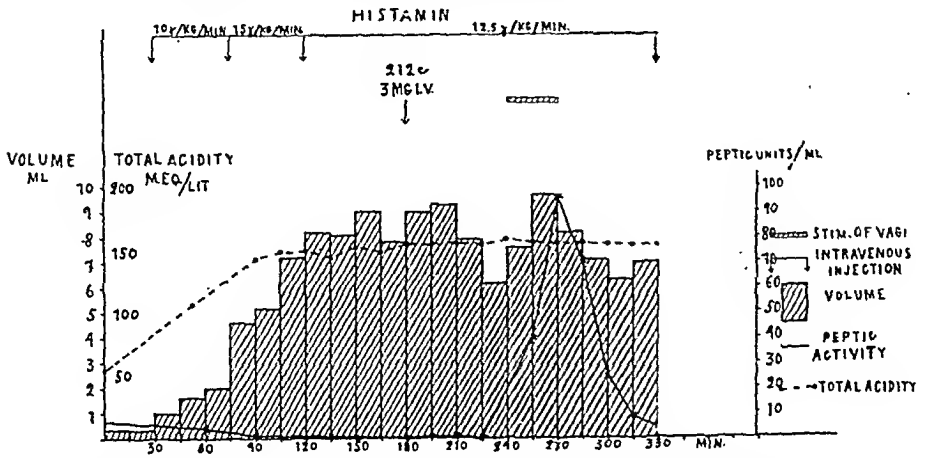


Fig. 2. Cat 3.7 kg. Peptic secretion after intravenous injection of a pyloric preparation, and after vagal stimulation. A steady gastric secretion is maintained by continuous intravenous injection of histamine.

secretion of about 8 ml gastric juice. 2.2 mg of another preparation yielded a secretion of 7.5 ml. A repeated injection of the first material, now in the double dose, only evoked a secretion of 3.4 ml. Four hours later the administration of the same dose initiated a secretion of more than 11 ml.

As a report of our investigations on the peptic secretion after the injection of pyloric preparations has not been published elsewhere these will be presented here.

Determination of Peptic Activity.

ANSON and MIRSKY (1932) described a method for the estimation of peptic activity with hemoglobin as the protein substrate. According to these authors "Hemoglobin has been chosen as the protein substrate for the estimation of pepsin because it is easily prepared in large quantities, because it is rapidly digested, and because the rate in which it is digested by a given pepsin solution does not vary from one preparation to another". BEAZELL *et al.* (1938) and BUCHER and BEAZELL (1941) have modified the method, "in order to widen the range of peptic activity over which the method can be employed without it being necessary to resort to dilution of the more active samples".

We have adopted the method for the Pulfrich Photometer. By suitable changes in the concentrations of gastric juice and hemoglobin and other minor modifications the method has been found to be easily managed and to be well suited for our experiments on canine and feline gastric juice.

The principles of the method. An acidified hemoglobin solution is incubated with pepsin under standard conditions. After a definite time of digestion the enzymatic process is interrupted by precipitating the undigested hemoglobin with trichloracetic acid. The precipitate is filtered off. The intensity of the blue colour which phenol reagent gives with protein digestion products (tyrosine, tryptophane and cysteine) is taken as a gauge of the digestion of hemoglobin. In the photometer the light absorption is determined and matched against a standard curve obtained with tyrosine.

Definition of peptic activity. One unit of pepsin is defined as that quantity which, acting at 37° C. in the standard digestion mixture used, would liberate in 60 minutes a quantity of material soluble in trichloracetic acid, a quantity, which in the assay mixture is equivalent to one mg of tyrosine. The peptic activity is given in units per ml of gastric juice.

Reagents used.

Hemoglobin solution	10 %
Trichloracetic acid	4 %
HCl	N/6
NaOH	N 3.85
Phenol reagent according to FOLIN and CIOCALTEAU.	

The preparation of hemoglobin is carried out according to ANSON and MIRSKY, with the modification that instead of carbon monoxide, coal gas is bubbled through the blood. The concentration of hemoglobin was estimated by the Kjeldahl method, as modified by ANDERSEN and JENSEN (1923), the nitrogen content being taken as 17.7 %.

Accuracy of the method. According to NORTHROP the pH-optimum for digestion of hemoglobin is 2.2. The acidity of our acid hemoglobin solution was chosen to give a maximal rate of digestion. Within the concentrations of hemoglobin and gastric juice used by us the digestion rate of hemoglobin proved to be proportional to the pepsin concentration. Duplicate determinations were always carried out. In a series of 10 duplicate determinations the standard deviation amounted to 0.02 mg tyrosine. The lowest amount of tyrosine which could be determined was 0.05 mg. This means that when no peptic activity was observed, the gastric juice could be said to be practically pepsin-free.

Procedure of testing. To make up the acid solution of hemoglobin, 3 volumes of N/6 HCl are added to 2 volumes of 10 % hemoglobin solution. 5 ml of this mixture are pipetted into a test tube and heated in a water bath at 37° C. for 15 minutes. 0.2 ml of gastric juice are then added. After electrical stimulation of the vagi gastric juice is generally diluted 5 times, whereas histamine juice and other juice poor in pepsin are used undiluted. After 15 minutes the digestion is interrupted by adding 10 ml of 4 % trichloracetic acid, and after careful mixing the precipitate is filtered off. 3 ml of the filtrate are pipetted into a glass vessel containing 20 ml distilled water and 1 ml of 3.85 N NaOH and 1 ml of phenol reagent are added. Readings are made in the Pulfrich Photometer after 15 minutes. After this time no colour change occurs within 1—2 hours. Cuvette length 10 mm and filter 57 are used. As the gastric juice in itself gives a slight colour reaction with phenol reagent, all samples are checked against a compensation solution. This contains the same ingredients as the other samples except that the gastric juice is inactivated. The pepsin is destroyed by boiling 5 minutes in a water bath.

The peptic activity of the gastric juice secreted after the administration of pyloric preparations was always low, and declined during the course of the secretion to extremely low values. These facts indicated that the secretory agent did not stimulate the peptic glands. The peptic activity might be due to the washing out of preformed pepsin from the gastric glands. BJÖRKMAN, NORDÉN and UVNÄS (1943) showed that histamine did not stimulate the peptic glands of the cat. The initial peptic activity of the gastric juice secreted after histamine was probably due to a washing out of pepsin stagnated in the gastric glands. By continuous intravenous injection of histamine a gastric juice free from pepsin could be obtained. We made use of this fact. On cats histamine was injected intravenously to give a secretion of a

gastric juice free of pepsin. After this an amount of pyloric material known to evoke a copious secretion of gastric juice was injected intravenously. None of our preparations evoked any significant peptic secretion. Fig. 2 illustrates such an experiment. The peptic activity of the gastric juice secreted under the administration of histamine declined to scarcely detectable values. The intravenous injection of a pyloric preparation in amounts previously observed to be highly active (see fig. 1) does not influence the peptic secretion. In contrast to this there is the copious secretion of pepsin during electrical stimulation of the vagi (for the technique see BJÖRKMAN and *co-workers*).

Crude pyloric preparations from pigs precipitated from HCl-extracts by trichloracetic acid slightly but definitely stimulated the peptic secretion. Now and then we observed that preparations prepared by the trichloracetic acid method of MUNCH-PETERSEN *et al.* caused a slight increase of the pepsin secretion.

Comments.

The investigations have confirmed previous reports from this laboratory on the distribution and properties of the gastric secretory excitant. Active material was obtained only from the pyloric mucosa. The active principle is a protein-like substance isoelectrically precipitable at a pH of about 4—5.5. It specifically stimulates the HCl glands of the gastric mucosa. These facts indicate, as thoroughly discussed in previous papers, that the secretory principle is identical with the gastric hormone, "gastrin".

Is there a Pyloric Principle which Stimulates Peptic Secretion?

PAVLOV has shown that the peptic activity of the gastric juice varies with the composition of the food. On dogs with isolated gastric pouches CAMPBELL (1934) observed that the peptic activity of the juice from the pouches fluctuated in relation to the percentage of lysine of the food introduced into the main stomachs. ZELIONY and SAVITSCH (1914) and SAVITSCH (1922) claimed that mechanical stimulation of the mucosa of an isolated pyloric pouch caused an augmented secretion of pepsin in the main stomach. These facts may indicate the existence of a humoral mechanism instrumental in the stimulation of peptic secretion during the gastric phase of secretion.

We have found that our isoelectrically precipitated preparations do not affect the pepsin secretion. Crude preparations, only precipitated from HCl-mucosal extracts by trichloracetic acid, slightly but definitely augmented the peptic output, however. Now and then we observed that preparations purified according to the trichloracetic acid method of MUNCH-PETERSEN and *co-workers* caused a slight increase in the peptic output. These observations may indicate that there is a principle in the pyloric mucosa which stimulates the peptic cells. HARPER and RAPER (1943) claim that they have found in the duodenal mucosa a principle of protein nature which selectively activates the enzyme-producing pancreatic cells. This factor, by the authors named pancreozymin, is supposed to secundate the secretine in the humoral regulation of the pancreatic secretion. *Per analogiam* it might be supposed that a similar principle, localized in the pyloric mucosa, would secundate the "gastrin" in the humoral control of the gastric secretion.

Is there an Inhibitory Factor in the Pyloric Mucosa of Pigs?

Fat inhibits the gastric secretion by the liberation of a hormone from the duodenal mucosa (LIM, 1933 and others). An inhibitory substance, enterogastrone, has been isolated from this mucosal region (CRAY, BRADLAY and IVY 1937 and others). The active principle is of protein nature and is precipitable by trichloracetic acid, NaCl and tannic acid.

WILHELMJ *et al.* (1936) from their works on dogs assume that gastric secretion is controlled by intragastric inhibitory processes initiated when the acidity of the gastric contents approaches 0.06 normal. At a normality of the gastric contents of 0.1 the gastric secretion is said to be completely or very markedly inhibited. The question whether the intragastric inhibitory mechanism is of reflex or humoral nature is left unanswered.

TEORELL (1933) is of the opinion that the acidity of the gastric content is regulated by a diffusion process. From studies on the cat he believes that hydrocloric acid diffuses out of the stomach through the mucosal wall, while neutral chloride passes into the gastric cavity. He believes that there normally exists across the gastric mucosa an exchange of hydrogen-ion from the gastric juice and sodium-ion from the tissue fluid or blood. WILHELMJ,

O'Brien and Hill (1936), studying the secretion in whole stomach pouches in dogs, and Shay, Gershon-Cohen and Fels (1939) from studies on man, did not find support for an acid absorption mechanism.

The inhibitory effect of our preparations *may* be due to the presence of an inhibitory substance. A substance chemically related to entero-gastrone would probably be precipitated by the precipitants used by us.

Summary.

A gastric secretory principle is isolated from the pyloric mucosa of pigs by isoelectrical precipitation. The secretory excitant stimulates the HCl-glands but not the pepsin glands of the gastric mucosa. The possibility of the existence of an agent which stimulates the pepsin cells, and another which inhibits gastric secretion, is discussed.

References.

Anson, M. L., and A. E. Mirsky, J. gen. Physiol. 1936. *16*. 59.
Babkin, B. P., Amer. J. Dig. Dis. Nutr. 1934. *1*. 715.
Bauer, Å., and B. Uvnäs, Acta Physiol. Scand. 1943. *8*. 158.
Beazell, J. M., C. R. Schmidt, A. C. Ivy and J. F. Monoghan, Amer. J. Dig. Dis. Nutr. 1938—39. *5*. 107.
Björkman, G., Å. Nordén and B. Uvnäs, Acta Physiol. Scand. 1943. *6*. 108.
Bucher, G. R., and J. M. Beazell, Amer. J. Physiol. 1941, *133*, 230.
Campbell, J. (from Babkin 1934).
Gray, J. S., W. M. B. Bradlay and A. C. Ivy, Amer. J. Physiol. 1937. *118*. 463.
Harper, A. A., and H. S. Raper, J. Physiol. 1943. *102*. 115.
Lim, R. K. S., Quart. J. exp. Physiol. 1933. *33*. 264.
Munch-Petersen, J., G. Rönnow and B. Uvnäs, Acta Physiol. Scand. 1944. *7*. 289.
Savitsch, V. V. (from Babkin 1922).
Shay, H. J., Gershon-Cohen and S. S. Fels, Amer. J. Dig. Dis. Nutr. 1939. *6*. 361.
Teorell, T., Skand. Arch. Physiol., 1933, *66*. 225.
Uvnäs, B., Acta Physiol. Scand. 1942. *4*. Suppl. XIII.
— Ibidem, 1943. *6*. 97.
— Ibidem, 1943. *6*. 117.
Wilhelmj, C. M., F. T. O'Brien and F. C. Hill, Amer. J. Physiol. 1936. *115*. 9. 429.
Zeliony, G. P., and V. V. Savitsch, Soc. Biol. Paris 1914. *77*. 50.

From the Department of Medical Chemistry, University of Uppsala, Uppsala.

On the Purification of the Thiamin-inactivating Fish Factor.

By

GUNNAR ÅGREN.

Received 12 January 1945.

In a previous paper it was demonstrated that the enzymatical cleavage of the thiamin molecule into the pyrimidine and thiazole was probably a reduction reaction (ÅGREN 1945 a). The enzyme inactivated by dialysis at a suitable pH or by oxidation with hydrogen peroxide could be reactivated by incubation with glutathione. For a further study of the enzyme it was necessary to work with purified material. In the present paper a method of purification is described, and some of the properties of the partially purified enzyme are demonstrated.

Experimental.

The enzyme was prepared from the viscera collected under the same precautions as previously described (LIECK and ÅGREN, 1944). Solutions of the enzyme not immediately used were always stored at —15°, when necessary after neutralization to pH 7.4, since the rate of inactivation of the acid enzyme solutions was rather high, even in the frozen state. Protein nitrogen was determined according to HERRIOT (1938). All determinations of pH were made with a glass electrode.

Thiamin was determined by the method of MELNICK and FIELD (1938) with some slight modifications (LIECK and ÅGREN, 1944). When not otherwise stated 1 ml aliquots of a thiamin solution containing 700 γ of vitamin B_1 were incubated with 3 ml of fish extract, neutralized when necessary to pH 7.4, and 1 ml of 0.05 M phosphate buffer pH 7.4 for 2 hours at 40°. Subtraction of the number of microgram thiamin found in the digestion test from that of the control test gave the amount of thiamin destroyed by the enzyme. One unit was

defined as that amount of activity which under the above conditions would cause the disappearance of 1 micromole (1×10^{-6} mole) of thiamin. In practice a preliminary assay was made with several dilutions of the unknown solutions. The assay was then repeated using the amount calculated to contain about 1 unit of activity. The same unit was used by SEALOCK *et al.* (1943) in their work on the occurrence of the enzyme in different tissues.

Results.

In the previously used method of purification (ÅGREN 1945 a) the enzyme was mainly prepared by isoelectric fractionation. This method, however, did not always give reproducible results. After several trials the following method was finally adapted and has been used for large routine preparations of enzyme from different species of fish.

I. The finally ground and frozen viscera — liver, spleen, intestine and gills — were shaken for 1 hour at 0° with an equal volume of water, and centrifuged for 1 hour at 3,000 r. p. m. in a 4 litre cooled centrifuge. The centrifugate = 1.

II. No. 1 was acidified to pH 5.7 and again centrifuged for 1 hour. The centrifugate = 2.

III. No. 2 was brought to 0.6 saturation by the addition of solid ammonium sulphate.

Together with some coarse (Hyflow) Cel the precipitate was filtered on large Buchner funnels.

IV. The clear slightly yellow coloured filtrate was brought to 1.0 saturation by addition of solid ammonium sulphate and the precipitate centrifuged off. The precipitate solved in $^1/_{10}$ of the volume of No. 1 was neutralized and stored at — 15° when not immediately used. The solution = IV.

As far as possible the whole procedure was carried out at a low temperature, about 0°, and was finished within 10 hours. The results obtained when purifying the enzyme from 1—2 kg of viscera from the tench and the ide are given in Table 1.

Table 1.

The purification of the thiamin-destroying fish enzyme.

The numbers refer to the different steps of preparation deseribed in the text.

Species of fish	I				IV			
	Volume in ml	Total nitrogen in mg per ml	Activity in units per ml of solution	Aetivity in units per mg of nitrogen	Volume in ml	Total nitrogen in mg per ml	Aetivity in units per ml solution	Activity in units per mg N
Teneh ...	1 000	3.5	0.49	0.14	200	1.0	0.90	0.90
Ide.....	900	3.2	0.50	0.16	120	2.0	3.60	1.80

By the method outlined above the enzyme could be purified about 10 times without any loss of activity (the experiment with the ide).

In working out the method of preparation the intention also was to apply it to some species of fish where the presence of the enzyme could not be demonstrated by means of the chemical method (LIECK and ÅGREN), but where the enzyme activity had been identified by the Phycomyces test (WIKÉN and ÅGREN, 1945). It was possible that small amounts of enzyme activity after concentration and purification would be more easy detectable with the chemical method. Viscera from herring, Baltic herring and turbot were used. The last species of fish was not included in the series of fish investigated by LIECK and ÅGREN, but a direct water extract of the viscera (1 : 1) did not destroy any vitamin during 24 hours of incubation at 40°. Viscera from the three species of fish were prepared according to the method given above. The precipitate obtained by saturation with ammonium sulphate was solved and used for analysis. The solutions usually contained about 2 mg of protein nitrogen per ml. Neutralized samples of 3 ml were incubated as usual with 1 ml of buffer and 1 ml of vitamin for 24 hours, without any signs of enzymatical inactivation of B_1. An attempt was made to reactivate possibly inactivated enzyme by addition of 5 mg of glutathione per 3 ml of fish extracts which only contained about 0.4 mg of protein nitrogen per ml. No reactivation could be observed. When 700 γ of vitamin was incubated for 2 hours with 5 mg glutathione but without any protein present, about 50 per cent of the vitamin was destroyed. Thus in a solution containing about 0.25 per cent of inactivable protein the glutathione effect was inhibited.

Next, an attempt was made at further purification of the ammonium sulphate fractionated enzyme. Lead acetate was used in conditions similar to those which had been tried with advantage in the purification of the aminopolypeptidase (ÅGREN, 1945 b). This enzyme in some respects recalled the fish enzyme. Both behaved as albumins when precipitated with ammonium sulphate and the isoelectric points of the two enzymes were not far from each other. In the experiments the purified fish enzyme was diluted so as to contain 0.8 mg of protein nitrogen per ml and then precipitated with 0.3, 0.5 and 0.7 ml of 0.5 N lead acetate per 10 ml of enzyme solution. Only small precipitates were obtained and the activity remained in the centrifugates.

From previous experience it was known that a loss of enzyme

activity easily occurred when alcohol or acetone fractionation was used (LIECK and ÅGREN). An attempt was therefore made to purify the enzyme through repeated fractionation with ammonium sulphate at room temperature. To solutions of the purified enzyme containing 3.5 and 0.8 of protein nitrogen per ml solid ammonium sulphate was added until a distinct precipitate was obtained. This was centrifuged at 11,000 r. p. m. More solid salt was added to the centrifugate until a new precipitate appeared. The precipitates from the more concentrated enzyme solutions were solved in water to the volume of the original enzyme solution. The precipitates obtained from the diluted enzyme solution were solved in water to $^1/_4$ of the volume of the original enzyme solution. The enzyme activity of the different precipitates is given in Table 2.

Table 2.

Fractionated precipitation with ammonium sulphate of the purified fish enzyme.

The figures are the extinction values of the digestion tests (D. T.) and control tests (C. T.) obtained as described in the experimental section.

Protein nitrogen in mg per ml	Degree of saturation with ammonium sulphate											
	0.30		0.50		0.60		0.70		0.80		0.90	
	D. T.	C. T.	D. T.	C. T.	D. T.	C. T.	D. T.	C. T.	D. T.	C. T.	D. T.	C. T.
3.5....	0.26	0.28	0	0.25	0	0.25	—	—	0.06	0.26	0.23	0.27
0.8....	0.27	0.27	0.19	0.25	0.08	0.26	0	0.23	0.10	0.25	0.25	0.26

In both series the activity was precipitated over a rather broad range of salt concentration, thus indicating that there were several protein components in the solutions. Calculating per mg of nitrogen a purification was obtained but the yield was low.

An enzyme solution containing 0.8 mg of protein nitrogen per ml was also precipitated with ammonium sulphate at 0°. The solid salt was added to 0.6 saturation and the centrifugate was stored at 0°. During the first week only inconsiderable precipitates were formed and centrifuged off. After 3 weeks a rather heavy precipitate had been formed. The probably amorphous precipitate was solved to the original volume of the extract and the enzyme activity determined direct and after activation with 5 mg of glutathione per 3 ml solution during 30 minutes at 0°.

The following extinction values were obtained:

Non-activated solution		Activated solution	
Digestion test	Control test	Digestion test	Control test
0.20	0.30	0	0.32

Thus, it was possible to reactivate the spontaneously inactivated enzyme by glutathione. As demonstrated above the activity of glutathione *per se* on vitamin B_1 is inhibited by the presence of 0.25 per cent of inactivable protein. The concentration of protein in the reactivated enzyme solution was 0.30 per cent. This demonstrates the specifity of the reactivation reaction.

The purified enzyme solution was also cataphoretically analyzed in the Tiselius apparatus at pH 6.02, 5.44, 5.02, 4.64 and 4.32. Purified enzyme solutions containing about 1.2 mg of protein nitrogen per ml were dialyzed for 6 hours at 0° during constant stirring against buffers of the usual ionic strength. In the apparatus three fractions were observed, which moved fairly parallel at the different pH. At pH 6.02 and 5.4 the migration was anodic, at pH 4.3 and 4.6 the migration was cathodic. At pH 5.0 the migration of the different fractions was inconsiderable. Accordingly, the chances to obtain a considerable purification of the enzyme through cataphoresis were not great. By dialysing at pH 4.6 and 4.3 a precipitate was formed, which could be solved in water at pH 7.4. Usually about 50 per cent of the protein nitrogen was precipitated. The activity of the precipitated and solved protein was rather low, containing between 0 and 15 per cent of the total activity. The values were not changed when reactivation with glutathione was tried. The activity remained in the centrifuged solutions. By this procedure the activity calculated per mg of protein nitrogen became nearly twice as great.

Discussion.

By the method described in the experimental section the vitamin B_1 destroying fish enzyme was purified about twenty times. The purified enzyme solutions were yellow coloured. As regards solubility in pure water and in solutions of ammonium sulphate, the enzyme behaved as an albumin. In a previous paper (LIECK and ÅGREN) it was observed that there was a parallelism between the amounts of enzyme and red colour of the fish viscera. As men-

tioned above the enzyme is not a hemin-protein. On the other hand a similar parallelism was observed in the present investigation between the yellow colour and the activity of the purified enzyme solutions, and in the cataphoretical experiments the yellow colour and the activity moved parallel to one another. Further investigations will decide if this circumstance is only a mere coincidence.

In a previous paper (ÅGREN 1945 a) it was demonstrated that the fish enzyme inactivated by dialysis or treatment with hydrogen peroxide could be reactivated with small amounts of glutathione. In the present paper it has been demonstrated that the enzyme inactivated in a third way, by storage at 0° for three weeks, could also be reactivated by small amounts of glutathione. This fact certainly strengthens the assumption that the enzymatical cleavage of the thiamin molecule is a reduction reaction. In the previous paper it was also discussed if glutathione *per se* could inactivate vitamin B_1 *in vivo*. The evidence obtained in the present investigation renders the possibility rather unlikely. The presence of 0.25 per cent of inactivable protein effectively inhibited the direct glutathione effect on the vitamin. The possibility remained that the low output of aneurin in urine, obtained in balance experiments, depended on a change in the structure of the vitamin molecule during the time the urine is stored in the bladder. As a matter of fact, after incubation of 700 γ of vitamin with 4 ml of fresh, neutral urine for 2 hours at 40°, about 20 per cent of the thiamin had been changed into a form not detectable with the method of MELNICK and FIELD. Two explanations may be offered. Either the vitamin is reduced by substances acting like glutathione or is oxidized to aneurindisulphide. The latter possibility must be kept in mind after the recent discovery of MYRBECK *et al.* that aneurindisulphide occurs in yeast cells. In any case, the mere fact that the vitamin molecule may be changed during the storage of urine in the bladder obviously makes the accurature determination of vitamin B_1 in urine more difficult.

The method of purification will in the future be used for fractionation and tests with the Phycomyces method of different extracts, which according to chemical determinations do not contain the vitamin B_1-inactivating enzyme.

Summary. The thiamin-destroying fish enzyme has been purified about 20 times without considerable losses of activity.

The enzyme seems to belong to the group of albumins and the isoelectric point is close to pH 5.0. The enzyme has been inactivated in three different ways and then reactivated with glutathione. This strengthens the assumption that the enzymatical cleavage of the thiamin molecule is a reduction reaction.

The writer is indebted to the Ferrosan Corporation for a grant which supported the present investigation. He further acknowledges his thanks to Miss Ahlquist and Mr Eklund for valuable assistance throughout the investigation.

References.

HERRIOT, R. M., J. Gen. Physiol., 1944, *21*. 501.
LIECK, H., and G. ÅGREN, Acta Physiol. Scand., 1944, *8*. 203.
MELNICK, D., and H. FIELD, J. Biol. Chem., 1938, *127*. 515.
MYRBECK, K., VALLIN, I. and C. E. LUNDGREN, Svensk Kem. Tidskr. 1944. *56*. 296.
SEALOCK, R. R., LIVERMORE, A. H. and C. A. EVANS, J. Amer. Chem. Soc. 1943, *65*. 935.
WIKÉN, T., and G. ÅGREN, Arkiv för botanik, 1945. In press.
ÅGREN, G., Acta Physiol. Scand. 1945 a. In press.
—, Ibidem. 1945 b. In press.

From the Department of Physiology, Cambridge University, and the Physiological Institute at Helsinki University.

Reflex Motor Discharges in Single Nerve Fibres of the Frog in Strychnine Poisoning and in other Convulsive States.

By

EEVA JALAVISTO.

Received 20 January 1945.

The highest frequency at which the mammalian myelinated nerve fibre can be made to discharge impulses (at 37° C) is on the average about 1,000 impulses per second though the limit is of course affected by the thickness of the nerve fibre and other factors. In natural conditions the frequencies met with are usually lower. Sensory stimulation can however, occasionally elicit impulses at an equally high frequency in sensory nerve fibres, the highest rate of discharge for the sensory nerve of the tooth, for example, being about 1,000 impulses per second (PFAFFMANN). On the other hand, the critical rate of discharge for the muscle proprioceptors is lower, only about 500 impulses per second (MATTHEWS 1933). In ordinary conditions the motor nerve cells never reach values even as high as that. In forced convulsive breathing, for example, values higher than 120 impulses per second are not met with, and in the most intense voluntary effort maximal frequency in the motor unit does not exceed 50—60 impulses per second (ADRIAN and BRONK, 1929, LINDSLEY 1935, SEYFFART 1938 and personal communication). In reflexes even values as low as these are not generally reached. ADRIAN and MORUZZI succeeded in registering the discharge of impulses in single fibres of the pyramidal tract and observed somewhat higher values in them, e. g. a limit of 150 impulses per second after intense sensory stimulation or 200 impulses per second if the animal is under

chloralose anaesthesia. These values do not either approach the highest values of frequency of impulses for sensory endings. Only one case is known in which the frequency of impulses in a motor cell reaches the maximum frequency for sensory nerve endings. If strychnine is applied on the motor cortex of a cat convulsions are caused during which surprisingly high unitary frequencies of impulses can be registered in the pyramidal tract fibres, namely values up to 1,000 imp/sec. (ADRIAN and MORUZZI 1939). As on the other hand strychnine does not in the least raise the critical frequency of impulses in the tactile endings (BRONK 1929) it equalises the highest frequencies for motor (pyramidal fibre) and sensory systems (tactile endings).

The values mentioned above concern the mammals only. No systematic investigation concerning the maximum rate of unitary discharges in normal motor reflexes of the frog seems to exist. The greatest frequencies for its sensory endings have in many cases been found to be about 250—300 imp/sec. (CATTEL and HOAGLAND 1939, MATTHEWS 1931). If account is taken of difference in temperature in experiments with frogs and with mammals (VAN'T HOFF's rule, according to which the velocity of most biological reactions is doubled with a rise in temperature of 10 degrees (centigrade)) the sensory frequency maximums, at least are the same for both. The only experiments concerning the frequency of ventral horn cells date from investigations by BARRON and MATTHEWS 1938. In their papers mention is made of the effect of a constant depolarising current applied on the ventral horn of the frog. A continuous flow of impulses could then be registered from the fibres of the ventral root, the frequency of which was determined by the current strength. As the current increased, the frequency rose until a value of 60 imp/sec. had been reached. A further increase in the strength of the current made impulsation irregular or abolished it completely. Evidently, therefore, in the frog too the highest "functional" frequencies of impulses differ greatly in the sensory and motor systems. This complies with other physiological characteristics of the motor system. The motor end plate of the muscle fibre, for example, cannot respond to a greater frequency than 200 imp/sec., so that innervation rhythms at a greater rate would be wasted, so to say (GÖPFERT and SCHÄFER 1938).

The frog is a very suitable animal for experiments, both for the registration of impulses in single nerve fibres and especially

for investigating the effects of strychnine, because, contrary to what is the case with mammals, it remains alive for a long time in spite of the most violent convulsions. On account of this the experiments were carried out on frogs. The main purpose of my experiments was to examine the critical frequency of motor impulses under "normal" conditions as well as under the action of strychnine.

Methods of Experiment.

The experiments were carried out on English frogs (Rana temporaria). In some of the experiments the brain of the frog was removed under ether anaesthesia to the level of the optic lobes (brainstem frog), in some the brain was crushed down to the spinal cord with forceps (spinal frog). In the third group of experiments the brain was not damaged, but the frog was under chloralose anaesthesia, which leaves the spinal reflexes unimpaired or makes them even more lively, but puts the higher nerve centres out of action (chloralose frog). All the strychnine experiments were carried out on curarised spinal frogs. Before curarisation one hind leg was tied, the sciatic nerve being left outside the ligature so that the circulation in the leg was interrupted, but the nervous connection remained. The dose of curare was measured to completely kill nervous response in the muscles in about three quarters of an hour, with the exception, of course, of the tied leg. It was thus possible to observe from the reactions of the tied leg when the convulsive state had been reached, and the degree of its intensity. In some of the experiments, contractions were caused with the aid of picrotoxin, in some by placing the frog on ice for some hours before experimenting. The preparation by means of which the impulses of the single nerve could be made apparent was simple though requiring considerable patience. Either the digital nerves to the interosseal muscles or the sciatic nerve were dissected out, bound at their periferal end with a thin silk thread and cut off periferally. After this, additional fibres were severed from the nerve by means of a thin needle and sharp scissors, until the electrogram showed that only one or two fibres remained in action. This was judged by the "all or nothing" reaction of nerve fibres, that is, by the constant spike height of the same functional unit. As to whether this conclusion is justified, we shall return later on page 12. The recording electrodes were silver-silver-chloride wires in glass tubes filled with Ringer solution, with a cotton thread fitted in. Between experiments the nerve fibres were held lying on the muscles or in a Ringer bath and were placed on the electrodes for recording only. In this way no special moist chamber was needed. The temperature during the experiments was 15—19° C. Registration of action potentials was performed with a resistance condenser coupled amplifier in connection with a Matthews oscillograph or in later experiments (*e. g.* fig. 9) in connection with a cathode ray oscillograph using the so-called transverse sweep method first published by WILSKA

(1939). The advantage of the method is that with but a small film consumption the clarity of the record is excellent.

In the strychnine experiments, 0.1 to 0.2 mg strychnine nitrate (1 to 1,000) was injected into the dorsal lymph space, which dose proved to be the most appropriate for causing strong and lasting convulsions.

The reflexes were elicited simply by touching or pinching the skin of the frog at some point. In experiments in which the frog had received no strychnine injection it was usually necessary to try several different points before the reflex affected the nerve fibre on the electrodes. Especially in the spinal frog, reflex irradiation is very small even though strong stimuli are used. In the brain-stem frog it is considerably greater and the registration of the reflex single fibre neurogram correspondingly easier.

Maximal Frequency of Impulses in Spinal Frog, the Brain-Stem Frog and the Chloralose Frog.

The interpretation of results obtained from a frog whose exitability has not been artificially increased with the use of strychnine, for instance, is not altogether simple. The maximal frequency of impulses cannot be ascertained in one experiment, since the frequency of discharge varies, with the strength of the stimulus for one thing. This difficulty does not appear in strychnine experiments, in which the least supraliminal stimulation results in a maximal response the frequency of which cannot be raised by a further increase in the strength of the stimulus. It is probable, however, that if the skin of the same animal is strongly stimulated at different points and the highest frequency of the impulse discharges thus caused is chosen, it can with considerable probability be considered the real maximal frequency of discharge for the ventral horn cell in reflexes. It should further be taken into consideration that maximal frequency of impulsation may vary in different nerve cells. Table I shows the maximal frequencies thus obtained. It indicates the number of preparations in which the maximal frequency falls within the limits of values mentioned in the vertical column. Frequency of discharge is taken as the average discharge for 1/6th of a second. It is seen from the table that *spinal, brain-stem and chloralose frogs appear to have a different representation in different frequency groups.* Thus the maximal frequency of impulses (the 1/6th sec. average) is mostly under 60 imp/sec. in the spinal frog, generally greater that 90 imp/sec. in the brain-stem frog and also over 60 imp/sec. in the chloralose frog. It is true that the number of experiments is really too small to permit any exact evaluation but sufficient to indicate the

Table 1.

Highest frequency values in different experimental groups. Frequency calculated as an average for 1/6th sec.

	Number of frequency values falling within different frequency ranges				Total
	< 30 imp/sec.	35—60 imp/sec.	65—90 imp/sec.	> 95 imp/sec.	
Spinal frog	2	11	2	1 (max. 100)	16
Brain-stem frog....	1	1	2	11 (max. 135)	15
Chloralose frog	0	1	4	3 (max. 110)	8

class of magnitude of the maximal frequency. If the greatest frequency of discharge is estimated in a different manner, i. e. by the shortest period between two successive impulses, (as its inverse value) it is also observed that the maximal frequency of discharge for the motoneuron of the spinal frog is mostly lower than for the brain-stem frog, but the difference is not as pronounced. Table 2 demonstrates this relation.

Table 2.

Highest frequency values in different experimental groups. Frequency calculated on the shortest period between two successive impulses (imp/sec.).

	Number of frequency values falling within different frequency ranges				Total
	< 60 imp/sec.	65—100 imp/sec.	105—140 imp/sec.	> 145 imp/sec.	
Spinal frog	3	7	5	2 (max. 250)[1]	17
Brain-stem frog....	1	0	8	8 (max. 160)	17
Chloralose frog	0	0	5	3 (max. 190)	8

It may be mentioned that the general behaviour of the spinal frog is quite different from that of the brain-stem frog. The reflexes of the brain-stem frog are evidently livelier and their

[1] A frequency as high as this was once unexpectedly found between two impulses in an ordinary reflex. No evidence could be found that the impulses belonged to the activity of different neurones.

irradiation is greater than the spinal frog's. What this difference is caused by, whether it is the result, perhaps, of some secondary factor, caused for instance, by a difference in the conditions of blood circulation, or whether the brain-stem frog is in a state corresponding to decerebrate rigidity in mammals cannot be explained for the time being. It should be mentioned, however, that SCHRIEVER in examining *chronic* spinal and brain-stem frogs, established the fact that the time relations of the summation were different in each case, indicating a greater exitability in the brain-stem frog.

In any case the fact remains that the highest values for the frequency of reflex discharges of the motoneuron vary at least to some degree as a result of other than toxic causes.

Maximal Frequency of Impulses in the Strychnine Frog.

Convulsions caused by strychnine are reflex by nature. In the absence of sensory stimuli or voluntary impulses no contraction occurs (POULSSON 1920). The smallest stimulus, however, the table being jarred for instance, or a weak blast of air, causes the most violent convulsions in the frog. The whole nerve action potential then shows a series of large potential groups, appearing at a frequency of about 5—10 times a second (HOFFMAN, VÉSZI). The activity of the whole spinal cord also appears to be synchronized in compliance with this rhythm as BREMER has shown in examining the potentials of the spinal cord. As a whole the action potential is lengthened (UMRATH). After a series of contractions the frog loses exitability for some time, but recovers again. The longer the cramps have lasted the longer is the period required for recovery. Toward the end the frog may be completely paralysed for as long as 24 hours, but even this total unresponsiveness may be passing, if the dose of strychnine is not fatal (POULSSON). The paralysing effect of strychnine does not appear to be localized in the motor part of the central nervous system since a stimulus directed to a point on the skin not previously stimulated causes violent convulsions, though stimulation elsewhere on "fatigued" areas remains completely without effect. Previous work has been carried out only on the action potential of the whole nerve, which however only shows a random interference of the activity of innumerable fibres. How the ventral horn cell reacts during strychnine convulsions is not known.

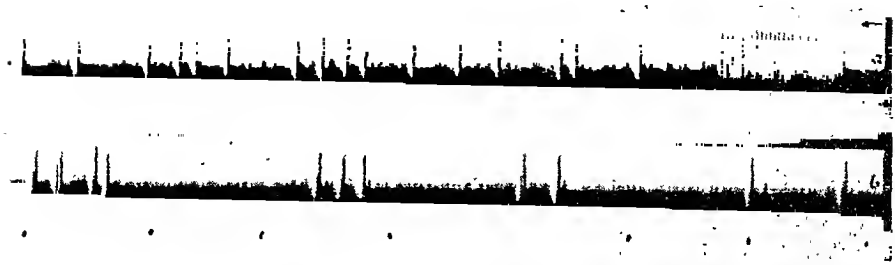


Fig. 1. A single fibre neurogram of the beginning of the convulsive stage in the strychnine frog. The lower record b. is taken 7 seconds after a. The dots on the lower margin mark 1/6th sec. To be read from right to left.

The frog does not, as the result of strychnine, immediately reach the typical convulsive stage. At first the exitability of the frog increases, the reflexes become very strong and lasting, but it is not yet in the typical opisthotonic posture, nor can as yet successive waves of convulsions be observed. It is comparatively difficult to make successful single fibre preparations during this stage, as the preparation cannot be completed before the injection of strychnine. This is due to the fact that in strychnine convulsions so many more nerve cells become active than in the ordinary spinal reflexes. Thus even though the preparation has shown only one nerve cell to be active before injection, the number called into action by the strychnine will be so great that it would be hardly possible to distinguish the individual impulses on the neurogram. In a few cases, however, I succeeded in recording a single fibre neurogram of this early stage (Fig. 1). The discharge comes from the fibres of the interosseal muscular nerve of the hind leg, the front leg on the same side having been lightly touched. a and b belong to the same neurogram, b showing the activity of the fibre 7 seconds after a. Typical of this early stage is the comparatively rapid frequency of discharge in the beginning (the highest frequency is 144 imp/sec.), followed by a long period of much slower but fairly regular impulsation. Only just before impulsation stops the impulses are grouped together forming series of 3—4 impulses (duration about 1/10th sec. with about 1/4th sec. between series). Often only one prolonged discharge of impulses takes place in the early stage. Fig. 2 a shows the variations in the frequency of such discharges. The duration of discharge and the frequency values vary considerably. The latter do not, however, in general rise above 200 imp/sec. The maximal frequency (calculated from the period between two successive im-

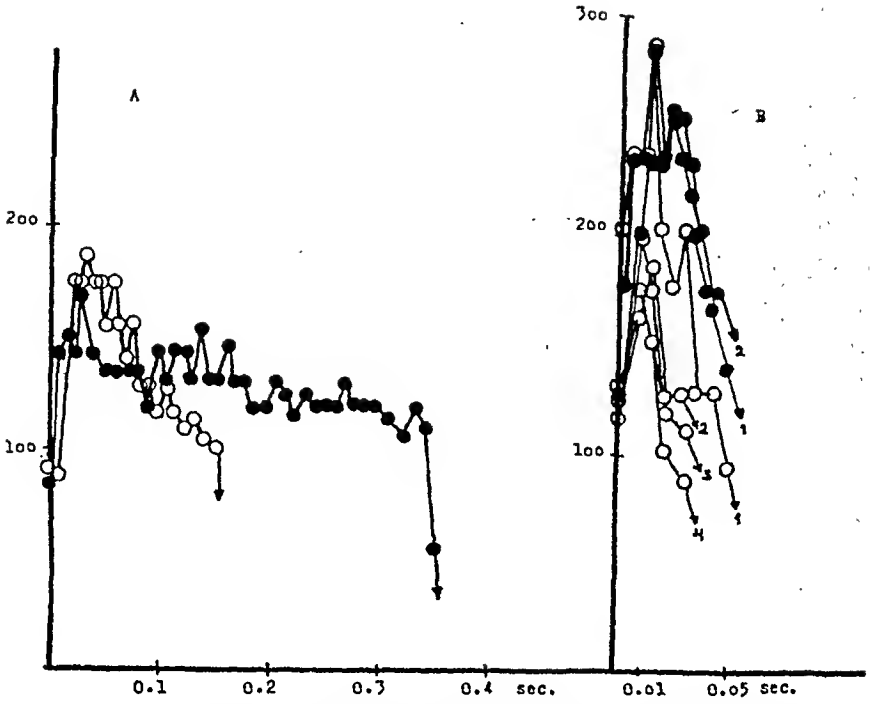


Fig. 2. The time-frequency curve of discharges of impulses in the strychnine frog, the ordinate being frequency of imp/sec. In the abscissa the time is measured in seconds from the beginning of impulsation. (It should be noted that the ordinate scale differs in fig. a and b.)

a) Single outbursts of impulses when the strychnine begins to take effect.
b) Series of discharge of impulses after the effects of strychnine have fully developed.

pulses) is usually attained only after several impulses have passed, and the resultant frequency-time curve is convex, beginning and ending abruptly. In later stages, when the strychnine has taken full effect, discharges of impulses preserve this form, but there are more groups, the frequency attains greater values and the duration of the series is reduced to only a fraction of what it had been. There are often about 5—8 series during $^1/_2$ to $1^1/_2$ seconds. Fig. 2 b gives the frequency-time curves of such discharges and fig. 3 contains several corresponding neurograms. It is to be noted that the whole series of impulse groups is now elicited by a single stimulus. The frequency-time curves of successive groups are on the whole similar in shape but the highest frequency value often falls in successive groups. Nevertheless, the frequency of discharge remains fairly high even to the last group. Usually 8—10 impulses form a group. A comparison

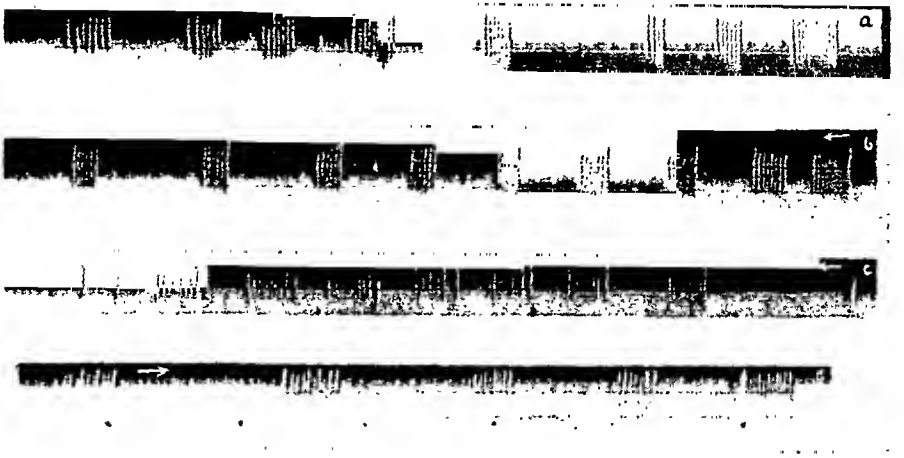


Fig. 3. Single fibre neurograms in fully developed strychninisation. Time, 1/6th sec. is marked on the lower margin. To be read from right to left. (In evaluating the amplitude of the impulses the shift in the base line should be taken into account.)

of these single fibre neurograms with the whole nerve action potential of earlier investigations (HOFFMANN, VÉSZI, FAHRENCAMP) shows that the occurrence of great and scarce potential waves of the latter and the frequency of high frequency impulse *groups* correspond. (Either is met with 5—8 or 5—10/sec.) It is thus fairly apparent, that the slow rhythms observed in the whole nerve action potential are caused by intermittent high frequency outbursts in a single fibre (Fig. 4 a). We have not, thus, to do with the interference of impulses from slowly (about 5—10 times per sec.) and almost synchronically impulsating fibres which would also give a similar total neurogram (Fig. 4 b).

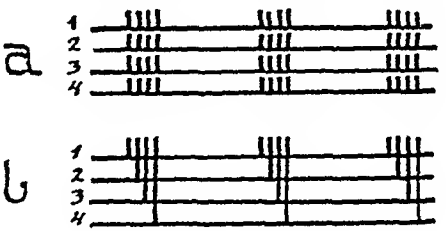


Fig. 4. Diagram demonstrating the probable structure of the whole nerve action potential in strychnine convulsions.

a) The correct interpretation indicated by our experiments.

b) Another possibility, not fitting the facts. 1—4 single nerve fibres with their impulses.

Table 3 gives a summary of the highest values for impulsation frequency in strychnine experiments. Frequency is calculated on the shortest period between two successive impulses. This seems to be the most suitable method because the maximal frequency determined in that way hardly varies in different experiments

Table 3.

The distribution of the highest frequency values in the ventral horn cell of the strychnine frog.

Highest frequency value imp/sec.	Occurence	
	In different experiments	In different preparations
< 150	1	1
151—200	27	4
201—250	54	13
251—300	38	10
> 300	6	3

with the same preparation. As table 3 indicates, *greater values than 300 imp/sec. are very rare, so that this value must be regarded as the critical frequency for the motor ventral horn cell.* In most cases the highest frequency is between 200 and 300 imp/sec. Table 4 (p. 326) gives individual maximal frequency values. It shows how regularly high values appear in fresh "unfatigued" preparations. When one considers that the end plate of the motor muscle fibre is unable to follow stimulation recurring at over 200 times per second, the frequencies observed must be considered surprisingly high. It should be noted that often when the neurogram showed higher frequencies the convulsions were much weaker in the tied leg than at the stage when the frequencies had not reached such high values. That one is really at the frequency limit of the motor nerve cell, is indicated by the fact that successive impulses could often be observed as coming within the relative refractory period caused by the preceding impulse (indicated by a reduced spike height).

In order to be able to compare the highest frequency observed in the frog with ADRIAN's and MORUZZI's highest values in experiments with mammals we must take into consideration a difference of temperature of about 20 degrees between the frog and mammal experiments. Assuming that VAN'T HOFF's rule (see introduction) can be applied, the frog values must be multipled by 4 in order to obtain a frequency of impulses which can be compared with mammal values. Thus 300 imp/sec. in frog experiments would correspond to a frequency of 1,200 imp/sec. in mammal experiments. As the coefficient of temperature is not known exactly, and the order of magnitude is the same in ADIAN's and MORUZZI's

experiments and my own, the values can be regarded as equivalent. This also lends support to the assumption that the registered highest value of 300 imp/sec. is the real maximal frequency limit, assuming that the impulses really are expressions of the activity of the same fibre.

In their work ADRIAN and MORUZZI consider the question of whether it is justifiable to conclude that impulses really belong to the same nerve fibre merely on the ground of their constant size on the neurogram. They were unable to offer any direct evidence in favour of this fairly generally accepted view. Though I was unable to demonstrate it directly with a histological preparation, the fact that often when the frequency of impulses was at its highest, the relative refractory state of the fibre became apparent as a reduction of the amplitude of the impulses might prove the unitary nature of the impulses recorded. For example, if the size of impulses be examined in a group of impulses whose frequencies vary, it can be fairly often noted that starting from a given minimal frequency value, amplitude and frequency change in opposite directions and regularly, so that the rise in frequency corresponds to the reduction in amplitude. MATTHEWS (1931) and lastly PFAFFMANN have noted a reduction in the amplitude of impulses of sensory endings, when the frequency is rising to this critical value. In fig. 3 b (first group), 3 c and 10 (cold frog), examples are given of the decrease of the amplitude of impulses as a result of high frequencies. Especially in the preparation 3 c the variations in amplitude appear systematically in a long series. Fig. 5 shows some diagrams in which both the amplitude of the impulse and frequency are taken as ordinates, the successive intervals between impulses representing the abscissa. Both curves show considerable reciprocity as long as the frequencies are high (part a of the curve), but at low frequencies an equally clear independence of each other (part b of the curve) (constancy of the height of the spike). The phenomenon might be regarded as a proof that the impulses in question are related to the activity of the same fibre.

The method of preparation we have used, where the fibres are gradually separated from one another, gives us some idea to what extent the activity of the nerve cells has been synchronized due to the effects of strychnine. As the impulses registered are conducted to the perifery, the slight asynchronism shown by the different fibres can be due to differing speed of propagation in the

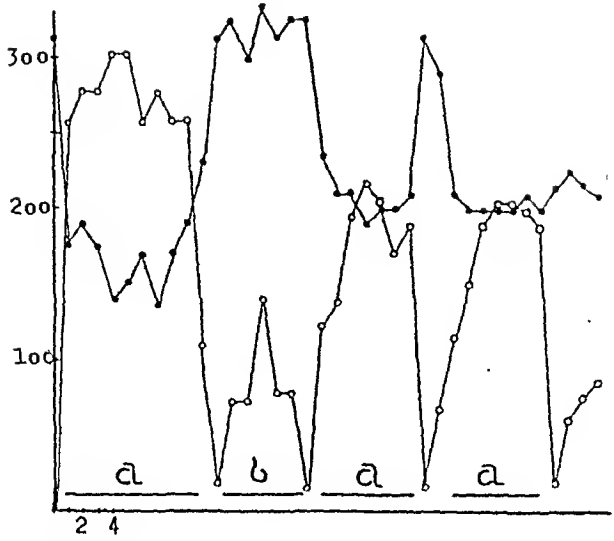


Fig. 5. Reciprocity of the frequency and the amplitude of the impulses, when frequency is so high that each successive impulse comes within the relative refractory phase of the preceding impulse. The ordinate is the frequency of imp/sec. (rings), and the amplitude of the impulse (dots), the scale is arbitrary. The abscissa on both curves is the successive time interval following impulses.

respective fibres. Thus experiments in which the impulses of two separate fibres have been successfully registered often show that the activity of the two neurons can be considered as being practically synchron. Examples of such cases are given in fig. 6. In all of them one can note that the grouping of impulses is the same in both fibres. The grouping is so exactly alike, that hardly a single impulse can be found between the groups. Nevertheless, one is not dealing with double impulses. This can be observed from the fact that (fig. 7 d) the distance between impulses belonging to different fibres decreases and increases and that at the moment when they appear simultaneously a greater potential (amplitude) is registered.

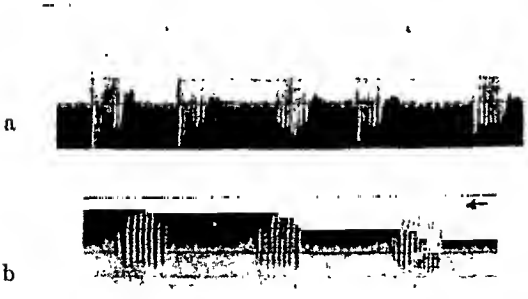


Fig. 6. Two fibre neurograms of strychnine frogs. To be read in the direction shown by the arrows. Time 1/6th sec.

In the strychnine frog the same motor neuron can be made to respond both by stimulation on the same and on the opposite

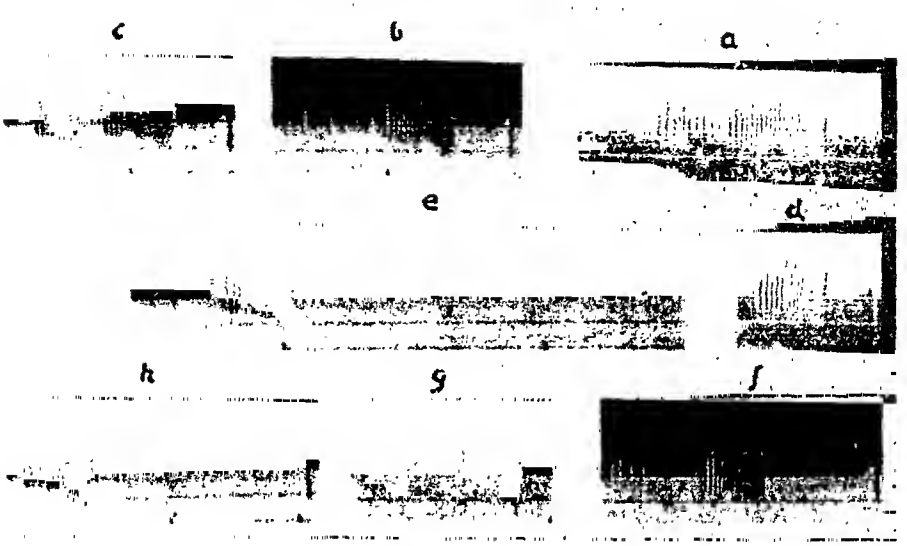


Fig. 7. Two fibre neurograms of the right leg of the strychnine frog. In a, b, and f the right front leg has been stimulated, in the others, c, d, g, and h, the left front leg. No appreciable difference can be noted between ipsi- and contralateral stimulations. Time 1/6th sec. To be read from right to left.

side, both on the hind and on the fore legs or the skin of the abdomen. This is, indeed, one of the typical characteristics of *the strychnine frog: The exceptional irradiation of the reflexes*, which would indicate that the one-way conduction in the synapses is invalidated and the effects of stimulation thus spread in all directions. Fig. 7 shows pairs of neurograms as a result of alternative stimulation on the right and left side. No difference can be found in the highest frequency values of the impulses elicited by stimulation of different sides.

Renewed successive stimulation changes the record in a typical manner, until the fibre in question ceases to respond. The number of impulse groups following stimulation decreases and the *maximal frequency of impulses* attained in each group *falls slightly*. Finally stimulation does not elicit more than a single discharge of impulses or not even that. *If the point of stimulation is thereupon changed, the result is again a group or several groups of impulses. It is worth noting here that the maximal frequency of impulses, which had gradually fallen returns to its original high value showing that fatigue had not at all affected the motor cell itself, but some point on the sensory side or internuncial part of the central nervous system.*

Table 4 shows some examples of such experiments. Stimulation is in all cases carried out by touching either the right or left front leg. In the first experiment 2. VI. 39 the front leg was stimulated 5 times at intervals of 1—3 seconds. The greatest frequency of impulses following stimulation in each of the first groups has been recorded and it shows a gradual fall until the fifth stimulation remains altogether without response. After this left leg was stimulated twice. In the first group the frequency of impulses can be seen to rise almost to its former height, but to fall after the next stimulation. In the preparation 6. VII. 39 stimulation is often followed by only a single group of impulses and repeated stimulation at the same point elicits no further response. The fall in frequency before unresponsiveness cannot therefore be demonstrated as clearly. However, these experiments too, show that *the fatigue of the sensory neurones is greater than that of the motor neurones*, since they cease completely to respond as the result of renewed stimulation, *the motor ventral horn cell still being able to respond maximally, i. e. with the highest possible frequency of impulses.*

Table 4.

Date	Ordinal number of stimulus applied at same point	Number of impulse groups following single stimulus	Highest frequency in each group	Period between stimuli in seconds	Region stimulated
2. VI. 39. (14)	I	3	*270* 217 201	3	Right front leg
	II	3	*283* 211 204	2	» » »
	III	2	*216* 193	$2^1/_2$	» » »
	IV	2	*188* 190	$1^1/_6$	» » »
	V	0	—	2	» » »
	I	6	*253* 200 232 271 225 246	2	Left » »
	II	3	*194* 181 177		» » »
6. VII. 39. (6 a)	I	1	*231*	3	Left » »
	II	0	—	$1^1/_6$	» » »
	III	0	—	$1^2/_6$	» » »
	I	1	*235*	$1^5/_6$	Right » »
	II	0	—	$1^5/_6$	» » »
	I	0	—	$1^1/_{42}$	» » »
	I	1	*250*		» » » but more proximal

Date	Ordinal number of stimulus applied at same point	Number of impulse groups following single stimulus	Highest frequency in each group	Period between stimuli in seconds	Region stimulated
6. VII. 39. (6 b)	I	1	*250*	$1^1/_2$	Right front leg
	II	0	—	2	» » »
	I	1	*263*	$1^5/_6$	Left » »
	II	1	263	2	» » »
	I	1	*263*	$1^5/_6$	Right » »
	II	0	—	$1^5/_6$	» » »
	I	1	*263*	$1^4/_6$	Left » »
	II	1	265	$2^1/_2$	» » »
	III	0	—	$1^4/_6$	» » »
	I	1	*260*	$1^4/_6$	Right » »
	II	1	129	$1^5/_6$	» » »
	I	1	*256*		Left » »
6. VII. 39. (9)	I	1	*303*		Right » »
	II	1	264		» » »
	I	1	*288*		Left » »
	II	1	271		» » »
	I	1	*262*		Right » »
	II	1	243		» » »
	I	1	*273*		Left » »
	II	1	261		» » »
6. VII. 39 (16)	I	1	*278*	2	Right » »
	II	1	274	$4^1/_3$	» » »
	III	1	207	$2^1/_2$	» » »
	IV	1	232	$2^1/_2$	» » »
	V	1	240	2	» » »
	I	1	*275*	$2^2/_3$	Left » »
	II	1	272	$2^2/_3$	» » »
	I	1	*274*		Right » »

The irradiation of the reflexes being so great, the question arises as to whether the impulses registered really are motor caused by the activity of the ventral horn cells. The fact that I never have succeeded in recording any impulsation in purely sensory nerves (nn. cutan. dorsi) comparable to those appearing in mixed nerves during strychnine convulsions even though I had made sure of the conductivity of the fibres by recording impulses of the same fibre elicited by tactile stimulation without changing the position of the electrodes, would appear to justify the assumption that the

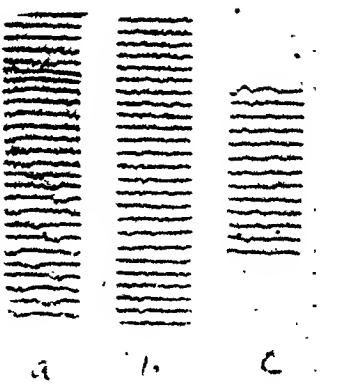


Fig. 8. Strychnine neurograms after reflex stimulation

a) from a mixed nerve, b) from cutaneous, sensory nerve, c) the same after the nerve had been killed by heat.

impulse groups observed are of motor origin. (Fig. 8.) BREMER also mentions having found in his strychninisation experiment on the spinal cord that no potential changes comparable to ventral root potentials did appear in the dorsal root during the convulsive phase.

Experiments Concerning other Convulsive States.

Picrotoxin neurograms. To enable a comparison of the peculiar form of impulsation in convulsions caused by strychnine with frogs found in other states of convulsion, I carried out some experiments with picrotoxin. According to ADRIAN and MORUZZI a picrotoxin crystal placed on the motor cortex of a cat causes strong convulsions, during which volleys of impulses can be registered in the pyramidal fibres, which resemble those caused by strychnine. My experiments, however, showed only a partial similarity between strychnine and picrotoxin neurograms. Even though the convulsions were strong the neurograms lacked the grouping of impulses characteristic of strychnine effects, nor did their frequency rise to as high a value (max. 180 imp/sec.). Fig. 9 shows examples of neurograms, which were recorded during a long series of convulsions. In the experiments picrotoxin was applied locally at the brain-stem, where, according to HEUBEL the strongest convulsions can be elicited. (In other cases picrotoxin was injected into the *dorsal lymphspace*, but the result did not differ from those where it was applied locally.) As can be noted from the figure, the impulses are not appreciably grouped, nor is the activity of the differ-

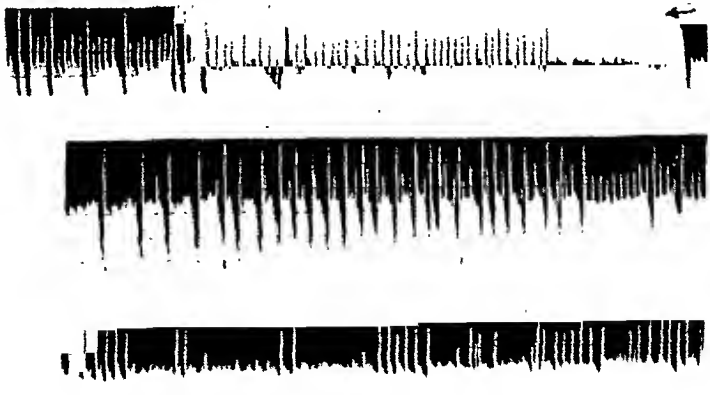


Fig. 9. Picrotoxin neurograms.

ent fibres synchronised to the extent it generally is in strychnine experiments. Only the lowest neurogram shows a slight tendency of impulses to form groups, and the record resembles an early stage of strychninisation. Even in this case the frequency of impulses is not great (150 imp/sec.). It should be mentioned that convulsive states caused by picrotoxin and strychnine resemble each other in that convulsions are in both cases reflex in nature, that the central sensory elements are blocked in the same way and that the refractoriness thus caused is passing. In picrotoxin experiments, however, final lasting unresponsiveness develops earlier than in strychninisation, which would indicate that the lower frequency and the lack of synchronisation of the picrotoxin neurogram did not result from too small a dose, or from the fact that the picrotoxin had not yet developed its full effects.

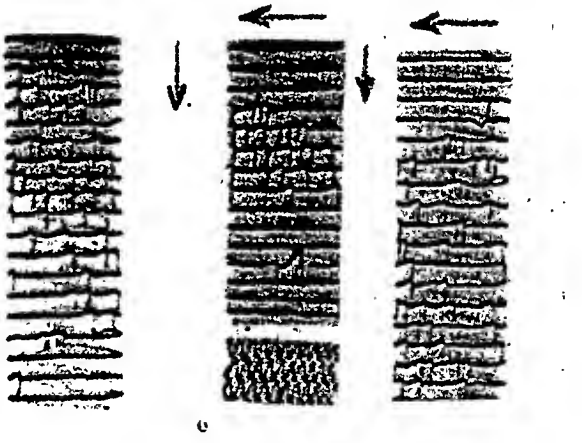


Fig. 10. Cold frog neurograms. To be read from right to left. The impulses appear as downward the stimulus artefact as upward deflections. Time 1/100 sec.

Cold convulsions. According to DE ALMEIDA cooling of the frog's spinal cord causes a state of convulsion. The same effect is obtained if the frog is kept on ice for some hours before the experiment. The convulsions (which again appear especially clearly in the brain-stem frog) externally completely resemble strychnine cramps — the frog acquires a rigid opisthotonic posture at the least touch. I recorded some single fibre neurograms of "cold frogs". Figure 10 shows some records of impulsation in the sciatic during convulsions elicited by tactile stimulation. The impulsation also resembles strychnine responses, the reflex (fig. 10 a & b) beginning with a few high frequency groups of impulses separated by clear "silent periods" and continuing less regular and slower just as is sometimes the case in strychnine tetanus. It is interesting to note that in this case too the frequency is quite at the highest limit for the fibre, for here too the amplitude

of the impulse can be clearly seen to decrease with the increase of frequency to over 200 imp/sec. Occasionally impulsation continues without interruption for long periods at a low frequency.

Discussion of Results.

We can compare the results described above not only with earlier investigations on the critical frequency of the motoneuron but also with investigations on its absolute refractory period. Since the absolute refractory phase means the shortest possible period between two neurone reactions (impulses), it can just as well be expressed as a frequency value, i. e. the inverse value of the *time period*, in which case it means the highest possible frequency of two successive impulses. According to BARRON and MATTHEWS the ventral horn cell of the spinal frog can impulsate regularly only at the rate of 60 imp/sec. The same value was obtained in our own experiments with the *spinal frog, i. e. in spinal* reflexes when frequency was determined during 1/6th of a second. On the other hand, the shortest period between two successive impulses found in our experiments and the absolute refractory phase of the spinal frog's motoneuron determined by the method of antidromic stimulation (UMRATH), are of the same magnitude. Also BREMER et al., on the basis of their summation experiments, come to the conclusion, that the "functional" refractory period of the spinal frog's motoneuron would be 8—12 msec. which would correspond to frequencies of 85—125 imp/sec. i. e. the values which are most common in our spinal frog experiments.

Fig. 11 gives a summary of these relations in the frog according to our own results and those of earlier investigators. The figure includes observations made on spinal, brain-stem, chloralose, cold as well as strychnine frogs. It shows clearly that *strychnine and cold make the motoneuron* discharge at a frequency which is as high as the critical frequency of impulse of the sensory endings. (250—300 imp/sec.) (*Both* in turn are somewhat below the corresponding value for the absolute refractory period of the nerve fibre.) This offers a striking contrast to the periferal action of strychnine which according to some investigators (BARTLEY and HEINBECKER, PEUGNET and COPPEE) lengthens the absolute refractory phase of the nerve fibre. If the effect of strychnine in raising the upper limit of the frequency of impulsation be interpreted as simply having a special effect on the refractory period

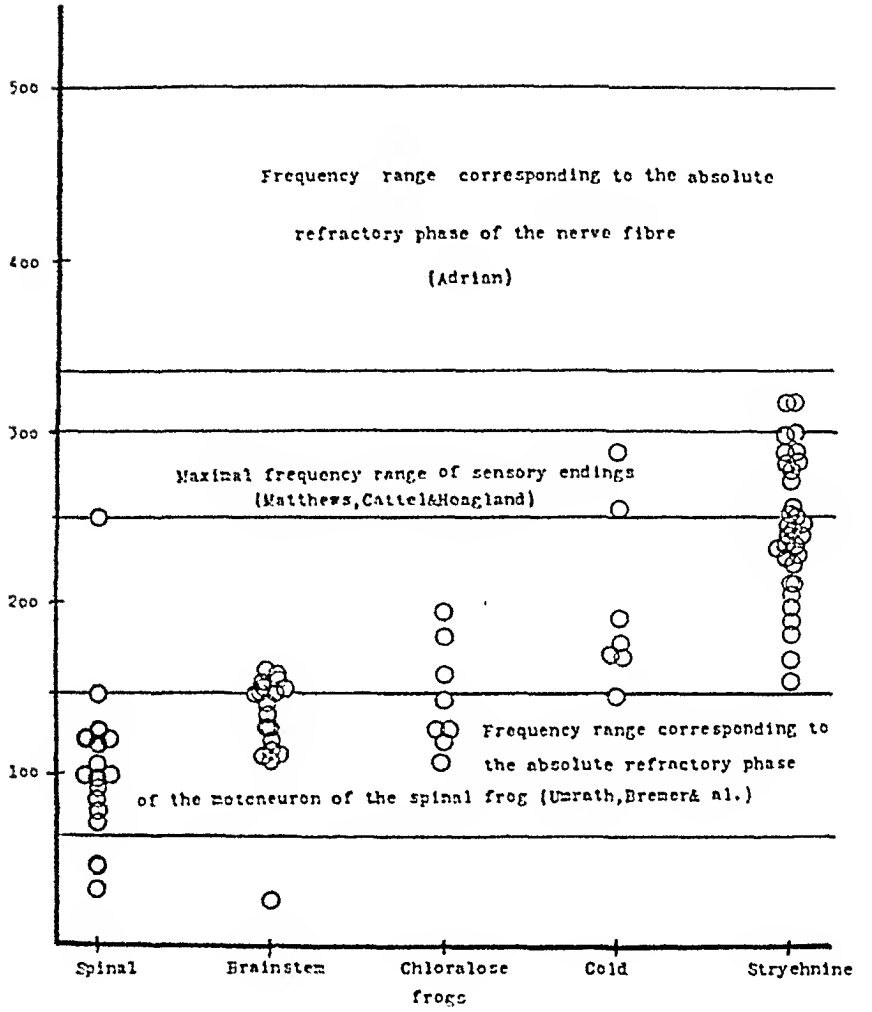


Fig. 11. Survey of results compared with values met with in literature. Ordinate frequency imp/sec. Abscissa: different experimental groups. In my own experiments frequencies are calculated on the shortest period between two successive impulses.

of the nerve cell it would be necessary to assume in addition that this effect would be directly opposite *in different parts of the cell* (in the nerve fibre and the neurone as a whole). This, however, does not appear probable. The following might better fit the facts.

Bartley and Heinbecker explain the action of strychnine to as a slowing down effect on the *accommodation* of the nerve fibres. If the activity of the cell, depolarisation, for example, does not return to resting value in the periods between impulses in strychninisation as quickly as normally, the remainder of the potential adds to the potential caused by the following impulse,

and the final result is a greater potential than if accommodation had been rapid. In support of their view the authors point out that, for instance, calcium, which quickens the accommodation of the nerve weakens the effect of strychnine. It is also very probable that cold too slows down accommodation, in which case the similarity between strychnine and cold frog neurograms would find a natural explanation. ADRIAN and MORUZZI explain the matter in the same way as BARTLEY and HEINBECKER. It should also be mentioned that slow depolarisation potentials much higher than normally can be recorded in the ventral root with strychnine (DUN, MATTHEWS, personal communication), which appear to correspond fully with the discharges of impulses in the nerves and which are, in the normal frog at least, known to correspond to the frequency of impulsation in the ventral horn cells, i. e. impulsation occurs only at a fixed level of the potential and becomes more frequent, the greater the slope and the higher the level of depolarisation is. If the high frequency of impulsation in the ventral horn cell were only the result of the greater depolarisation and slower accommodation of the cell, it should be possible to raise the frequency of impulses in the ventral horn cell of the ordinary spinal frog artificially with a depolarising current to as high a level as with strychnine. According to BARRON and MATTHEWS, this however, cannot be done: the ventral horn cell did not discharge at a frequency greater than 60 imp/sec. which generally is the maximal frequency of impulsation for the reflexes of the spinal frog too, even though the strength of the depolarising current was arbitrarily increased. One would therefore have to assume that some additional factor affects the frequency of impulsation in strychninisation.

ADRIAN refrains from treating the nature of the process which causes the depolarisation of the cell. On the other hand GESELL's theory of neurone activity is concerned with this point.

According to GESELL the frequency of impulsation of the neurone is determined by the potential difference caused by the metabolic gradient which arises between the dendritic part of the neuromembrane containing numerous synapses and the axon hillock with only scarce synapses when the synapses become activated. The resultant difference on the depolarisation potential increases with frequency of impulsation and number of activated synapses in the dendritic part and decreases with the frequency of impulses arriving in synapses at the axon hillock.

It would seem to me that GESELL's hypothesis complies strikingly well with the interpretation of some strychnine effects and my own results.

In the reflexes of the normal frog, in which irradiation is small, i. e. number of functioning synapses small, the frequencies in question are low compared with the frequencies of the reflexes of strychnine and cold frogs, with maximal irradiation of reflexes. Since strychnine and cold partly destroy the one-way conduction in the synapses (DUN, MATTHEWS) releasing new neurone chains into action, conditions are created allowing for high frequencies of impulsation (the number of activated synapses increases). This would be the case even if the accommodation in the cell had not been slowed down.

This would also offer a basis for settling the old argument about whether strychnine acts as a stimulant itself or not. According to DUSSER DE BARENNE the application of strychnine to the ventral horn cells alone is not enough to produce convulsions. Thus convulsions are caused only if both the ventral and dorsal parts of the spinal cord are affected by strychnine. Strychnine would therefore not appear to be exitatory in itself. ADRIAN and MORUZZI, however, succeeded in producing convulsions by applying strychnine to the motor cortex of the cat. This observation which appears to contradict the foregoing may be due to the peculiar structure of the cortex and to the latent activity always to be found in it. In the cortex even cells of the outer layer send out a vast amount of branching fibres and contain an abundance of synapses. The excitatory effect of strychnine could therefore be explained only by assuming an abolition of the one-way conduction to occur in the synapses which opens new chains of neurones to activity. More synapses in the pyramidal cells are in this way activated and the latent activity is thus transformed into strong convulsions with high frequency outbursts of impulses.

The observation made by SHERRINGTON, that strychnine has the effect of changing inhibitory reflexes to excitatory would find its natural explanation in GESELL's theory. As the number of active synapses increases at random due to the effects of strychnine and the density of the synapses is generally greater in the dendritic part of the cell than in the axon hillock, the inhibitory effects must lose ground, and the potential difference between the dendritic and hillock parts increases leading to excitation.

I wish to express my profound gratitude to Professor E. D. ADRIAN for his very valuable encouragement and all the kind guidance given to me during the first experimental stage of this investigation. Unfortunately because of the war I have not had the opportunity of discussing my interpretations of the results with him. I did not wish to publish my work earlier, in anticipation of this opportunity but with the prolongation of the war I do not feel I can put off its publication any further. The initial experiments were carried out together with Prof. MELVIN to whom also my best thanks. I am also very grateful to B. H. C. MATTHEWS for his many valuable advices.

The investigation has been supported by grants from the "Academia scientiarum Fennicae" and the "Societas Medicorum Fenniae 'Duodecim'".

Summary.

The maximal frequency (at room temperature) of reflex impulsation in single nerve fibres of the frog was examined. It was found to vary in different conditions being lowest (up to 125 imp/sec.) in normal reflexes of a spinal frog, probably somewhat higher in brain-stem and chloralose frogs and distinctly higher in convulsive states especially in strychnine cramps viz. up to 300 imp/sec.

The peculiar form of activity in single fibres under strychnine and cold convulsions was also investigated. It could be stated that during fully developed strychnine and cold cramps the single fibre neurogram consisted of high frequency outbursts of impulses appearing 5—10 times per sec., the groups being separated from each other by a more or less distinct silent period. The impulse groups in different fibres showed a considerable synchronism.

In the discussion it is pointed out that increased irradiation of reflexes e. g. in brain-stem or strychnine frogs compared with spinal, normal frogs usually corresponds to a raised "functional" frequency maximum of the motor nerve cell. As it is known that strychnine as well as cooling of the cord partly abolishes the one-way conduction in synapses leading to maximal irradiation of reflexes, the possibility to explain some other phenomena in convulsive states on this basis was considered. According to GESELL the number of impinging signals and of activated synapses at the dendritic part of the neuromembrane causes a po-

tential gradient between the dendritic and axon hillock parts of the neurone, that determines the degree of activity of a nerve cell. If this theory is applied on the strychnine effects, it might be possible to interpret the excitatory action of strychnine on motor cortex and the strychnine reversing of inhibitory reflexes to excitatory as results of the failure of one-way conduction in the cord caused by strychnine.

References.

Adrian, E. D., The basis of sensation. London 1928.
— The mechanism of nervous action. Philadelphia 1932.
— and D. W. Bronk, J. Physiol. 1929. *67.* 119.
— and G. Moruzzi, J. Physiol. 1939. *97.* 153.
Barron, D. H. and B. H. C. Matthews, J. Physiol. 1938. *92.* 276 and *94.* P 5.
Bartley, S. H. and P. Heinbecker, Amer. J. Physiol. 1939. *125.* 172.
Bremer, F., Arch. int. Physiol. 1941. *51.* 211.
—, V. Bounet and J. Moldaver, Arch. int. Physiol. 1941. *51.* 1.
Bronk, D. W., J. Physiol. 1929. *67.* 17.
Cattel, McK. and H. Hoagland, J. Physiol. 1931. *72.* 392.
de Almeida, M. O., C. R. Soc. Biol. Paris. 1936. *121.* 586.
Dun, Fwu, Tarng, J. Physiol. 1939. *95.* 41 P.
Dusser de Barenne, J. G., Physiol. Rev. 1933. *13.* 325.
Fahrencamp, K., Z. Biol. 1912. *59.* 426.
Fessard, A. and B. H. C. Matthews, J. Physiol. 1939. *95.* 39.
Göpfert, H. and H. Schaefer, Pflüg. Arch. ges. Physiol. 1938. *239.* 597.
Gesell, R., Ergebn. Physiol. 1940. *43.* 377.
Heubel quot. Trendelenburg in Heffters Hb. Pharmakol. Berlin 1920. *II.* 406.
Hoffmann, P., Arch. Anat. Physiol., Physiol. Abt. Suppl. 1910. 233.
Lindsley, B. Amer. J. Physiol. 1935. *114.* 90.
Matthews, B. H. C., J. Physiol. 1931. *71.* 64 and 1933. *78.* 1.
Pfaffmann, C. , J. Physiol. 1939. *97.* 207 and 220.
Peugnet, H. B. and G. E. Coppee, Amer. J. Physiol. 1936. *116.* 120.
Poulsson, E., Heffters Hb. Pharmakol. Berlin 1920. *II.* 1. 356.
Schriever, H., Z. Biol. 1933. *94.* 285 and 1934. *95.* 105.
Seyffart, H., Acta psych. 13: Fasc. 3.
Umrath, K., Pflüg. Arch. ges. Physiol. 1934. *233.* 257.
Vészi, J., Z. allg. Physiol. 1912. *12.* 358 and 1913. *15.* 245.

From the Department of Physiology, University of Lund.

The Potentiating Effect of Neostigmine[1] and Eserine on the Action of Morphine as Measured by the Straub-Herrmann Reaction.

By

THURE WRAMNER.

Received 6 February 1945.

When morphine is injected subcutaneously in mice the tail is erected in a characteristic S-shaped manner, and with proper doses the tail is kept vertical or curved towards the back (STRAUB 1911). This reaction was investigated in detail by HERRMANN (1912), and is in German papers often referred to as the Straub-Herrmann reaction.

HERRMANN considered a "catatonic" condition to cause the reaction, but did not explain in what manner. v. LEERSUM (1918) assumed that the reaction is caused by spasm in the urinary bladder and the rectum due to a direct influence of morphine on the spinal parasympathetic centres. MACHT (1920) observed that morphine elicited spastic contractions on the isolated urinary bladder and its sphincters, and presumed that the same effects should occur in the intact animal. MACHT suggested that the Straub-Herrmann reaction was due to the action of morphine on the urinary bladder and the rectum. HEINEKAMP (1921), however, obtained the reaction on white mice after removal of the urinary bladder and the rectum with its sphincters. Moreover, he was able to elicit this reaction with several drugs which stimulated spinal motor centres. He concluded that the Straub-Herrmann reaction is caused by stimulation of nerve cells in the spinal cord.

[1] Neostigmine (the dimethylcarbamic ester of 3-hydroxyphenyl-trimethylammonium methylsulphate) manufactured by A. B. Leo, Hälsingborg, is chemically and pharmacologically identical with Prostigmine.

Fig. 1. Three of the 25 mice injected with 0.01 mg morphine hydrochloride.

Fig. 2. Three of the 25 mice injected with 0.01 mg morphine hydrochloride + 0.0005 mg neostigmine.

Fig. 3. Three of the 25 mice injected with 0.01 mg morphine hydrochloride + + 0.0005 mg neostigmine + 0.002 mg atropine sulphate.

Fig. 4. Three of the 20 mice injected with 0.01 mg morphine hydrochloride + + 0.0004 mg eserine salicylate.

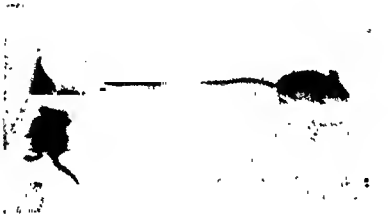


Fig. 5. Three of the 25 mice injected with 0.01 mg morphine hydrochloride + + 0.0004 mg eserine salicylate + 0.002 mg atropine sulphate.

Recent observations indicate that morphine in some way or other is linked up with a cholinergic mechanism. SLAUGHTER and GROSS (1940) found that eserine potentiates the motor action of morphine on the intestines in dogs and the depressor effect on the blood pressure in cats. Subcutaneous injection of 5 mg morphine per kg body-weight in dogs causes an inhibition of the serum choline esterase (SLAUGHTER and LACKEY 1940). Neostigmine as well as eserine potentiates the pain threshold raising effect of morphine in man (FLODMARK and WRAMNER 1944). JUUL (1939) has demonstrated that the Straub-Herrmann reaction is abolished by atropine.

Methodical.

Fifty white mice with a body-weight between 17—24 g were used. The administration of the examined substances was made by subcutaneous injections in the skin of the back. Every mouse was used for several tests but between each series of injections there was, for every mouse, an interval of at least one week. The temperature in the room during the tests was constant. The positive reactions are classified in three degrees. The reaction is considered as weak (+) when the tail is raised at about 40° at most, as medium (++) when the tail remains in an approximately vertical position, and as strong (+++) when the tail is curved over the back of the mouse.

Results.

Morphine hydrochloride was injected into 20 mice in a dose of 0.5 mg. All the mice showed medium or strong reactions. When the tails were erected the mice were given inhalations of amyl-nitrite in order to relax spastic smooth muscles. Although this drug was applied until the tail turned red from vasodilatation it did not diminish the erection.

The results are summed up in the following table.

Table.

Substances injected subcutaneously in each mouse	Number of mice injected	Percentage of mice which showed the reaction:			
		Negative	+	++	+++
0.01 mg morphine hydrochloride	25	68	32	0	0
0.01 mg morphine hydrochloride + + 0.0005 mg neostigmine	25	0	32	64	4
0.01 mg morphine hydrochloride + + 0.0005 mg neostigmine + 0.002 mg atropine sulphate	25	68	28	4	0
0.01 mg morphine hydrochloride + + 0.0004 mg eserine salicylate ...	20	0	35	50	15
0.01 mg morphine hydrochloride + + 0.0004 mg eserine salicylate + + 0.002 mg atropine sulphate. ...	25	76	24	0	0
0.0005 mg neostigmine	10	100	0	0	0
0.0004 mg eserine salicylate	10	100	0	0	0
0.002 mg atropine sulphate	10	100	0	0	0

Three of the mice in each of the five first groups in the table above have been photographed in order to illustrate the different types of reaction.

Discussion.

The observation that inhalation of amylnitrite does not abolish the Straub-Herrmann reaction opposes the assumption that this reaction is due to spasm in smooth muscle. Contractions of the striped muscles of the tail are likely to cause the reaction. For the smallest dose of morphine required for this effect, HERRMANN gives 0.01 mg morphine hydrochloride, whereas other investigators found larger amounts necessary. JUUL (1939) found that 0.01 mg morphine hydrochloride caused a positive reaction only in 30 % of 20 mice. In this series 32 % of 25 mice showed a positive reaction with 0.01 mg; which agrees with JUUL.

The table shows that neostigmine as well as eserine potentiates the effect of morphine. In previous experiments from this laboratory it was demonstrated that neostigmine and eserine potentiate the action of morphine on the pain threshold in man (FLODMARK and WRAMNER 1944). JUUL (1939) showed that large doses of atropine entirely or partly annulled the action of morphine on the tail of mice. In these experiments on mice a small dose of atropine greatly reduces the potentiating effect of neostigmine and eserine on morphine.

The potentiation described in this paper, the marked reduction of the potentiating effect by atropine, and the suppression of the Straub-Herrmann reaction by atropine, strongly suggest that morphine interferes with a mechanism of cholinergic transmission which is facilitated and enhanced by neostigmine and eserine.

Summary.

1. Neostigmine potentiates the effect of morphine in the Straub-Herrmann reaction.

2. Eserine potentiates the effect of morphine in the Straub-Herrmann reaction.

3. Atropine annuls the potentiating effect of neostigmine and eserine.

4. The findings are in agreement with the theory that morphine partly acts through a cholinergic mechanism.

References.

FLODMARK, S., and T. WRAMNER, Acta Physiol. Scand. 1945. *9*. 88.
HEINEKAMP, W. J. R., J. Pharmacol. 1922. *20*. 107.
HERRMANN, O., Biochem. Z. 1912. *39*. 216.
JUUL, A., Arch. int. Pharmacodyn. 1939. *62*. 69.
v. LEERSUM, E. C., Arch. néerl. Physiol. 1918. *2*. 689.
MACHT, D. I., Proc. Soc. Exp. Biol., N. Y. 1920. *17*. 100.
SLAUGHTER, D., and E. G. GROSS, J. Pharmacol. 1940. *68*. 96.
SLAUGHTER, D., and R. W. LACKEY, Proc. Soc. Exp. Biol., N. Y. 1940. *45*. 8.
STRAUB, W., Dtsch. med. Wschr. 1911. *37*. 1462.

Aus der pharmakologischen Abteilung des Karolinischen Instituts zu Stockholm.

Gleichzeitige Bestimmung von Äther und Kohlendioxyd in der Atmungsluft.

Von

PAUL LINDE.

Eingegangen am 12. Februar 1945.

Bei gleichzeitigem Vorkommen von Äther und Kohlendioxyd in einer Luftprobe ist es mit der gewöhnlichen Absorptionsanalyse nur möglich den Äthergehalt zuverlässig zu messen, während die Kohlensäureanalyse unrichtige Resultate gibt.

Von GRAMÉN ist im Jahre 1922 ein Verfahren veröffentlicht worden, den Äthergehalt in der Atemluft zu bestimmen. In einem speziellen Gefässsystem wird die Luftprobe durch eine genau abgemessene Menge eines Gemisches von Kaliumdichromat und konz. Schwefelsäure geblasen. Vom Äther wird das Dichromat reduziert, und aus der Reduktion berechnet man nach Titrieren die Äthermenge. Dieselbe Methode ist von LILJESTRAND und LINDE (1930) zur Messung des Alkoholgehalts in der Atemluft nach Alkoholeinnahme angewendet worden. Da indessen für die Analyse Luftproben von einem bis zwei Liter nötig sind, ist die Methode unbequem, wenn man z. B. kleine Experimenttiere benutzt. Da ausserdem das Dichromatgemisch Kohlendioxyd absorbiert (s. unten), ist es unmöglich, in derselben Luftprobe den Kohlendioxydgehalt zu bestimmen. Umgekehrt kann man aus analogen Gründen auch keine Ätherbestimmung nach einer Kohlendioxydanalyse machen.

KRAUSE (1923) meint, dass konz. Schwefelsäure Äther quantitativ absorbiert, ohne dass der Vorgang von gleichzeitig anwesendem Kohlendioxyd gestört wird. Meine Versuche zeigen indessen, dass dies nicht stichhältig ist. Sein Irrtum lässt sich vermutlich dadurch erklären, dass sein Apparat, der eigentlich als Übungsapparat för Studentenlaboratorien bestimmt war, und kein Manometer hatte, nur Annäherungswerte gab.

In meinen Versuchen wurden sämtliche Gasanalysen in HALDANES Apparat für volumetrische Gasmessung gemacht. Alle Probeentnahmen wurden mit queeksilbergefüllten Glaspipetten bewerkstelligt, die etwa 20 Kubikzentimeter fassen.

Zuerst wurde geprüft, wie sich die übliche kohlensäureabsorbierende Lösung (20 % KOH in gesättigter NaCl-Lösung) gegen Äther verhält. Mit Äther versetzte atmosphärische Luft (»Aussenluft«) wurde in den Apparat eingeführt. Nach den ersten fünfzehn Absorbierungen hatte das Gasvolumen 12.9 % abgenommen, nach dreissig war es mit 13.2 % reduziert und erst nach vierzig konstant 13.5 %. Unzweideutig nimmt die Lauge Äther bis zu einem gewissen Gleichgewichtszustande auf. Folglich kann eine richtige Kohlendioxydbestimmung in einer Ätherhaltigen Luftprobe erst gemacht werden, nachdem der Äther weggeschafft worden ist.

Danach wurden andere ätherabsorbierende Mittel versucht. Ein Diehromatschwefelsäuregemisch wurde geprüft, worin die Diehromatkonzentration n/30 und die Schwefelsäure 60—40 % ausmachte. (In schwächeren Säurekonzentrationen geschieht die Reduktion des Äthers langsam.) Luft mit Zusatz von 20 % Kohlendioxyd wurde untersucht. Nach vierzig Absorbierungen war das Gasvolumen mit 4.0 % vermindert, und blieb danach konstant. Die Reduktion entsprach also in diesem Falle einem Fünftel der Gesamtmenge des Kohlendioxyds. Das Diehromatgemisch war wie bei allen Äther- und Alkoholanalysen mehr als 24 Stunden alt. Mit neubereitetem Gemisch wurde das Gasvolumen auch nach hundert Absorptionen nicht konstant.

Das gute Ätherlösungsmittel, flüssiges Paraffin, zeigte sich hier unanwendbar. Es verschlang Luft, Kohlendioxyd und Äther und konnte auch gelegentlich Gas abgeben.

Konz. Schwefelsäure (nach KRUSE) wurde geprüft, und dazu wurde »Acid. sulphurie. cone. 'Pro Analysi' (KAHLBAUM)« verwendet. Aus einem Luftgemisch mit 20 % Kohlendioxyd wurde bei wiederholten Versuchen etwa 1½ % absorbiert. Aussenluft ohne Kohlendioxydzusatz verlor bei demselben Verfahren nur 0.2 %. Die Schwefelsäure löst also Kohlendioxyd in einer nicht zu vernachlässigenden Menge.

Nach diesen Erfahrungen schien es aussichtsreicher, dass das vorgesetzte Ziel mit Verbrennungsanalyse erreichbar sein würde. Ätherverbrennung dürfte von dem in der Probe anwesenden Kohlendioxyd nicht beeinflusst werden. Wenn man Äther verbrennt und danach Kohlendioxydbestimmung macht und dabei die gefundene Kohlendioxydmenge der berechneten gleich ist, bekommt man ein gewisses Kriterium der Methode und einen Beweis dafür, dass reiner Äther verbrannt worden ist. In einer Atemluftprobe findet man selbstverständlich einen Überschuss an Kohlendioxyd, welcher der in der Probe enthaltenen ursprünglichen Kohlensäure entspricht.

Äthyläther wird nach dieser Gleichung verbrannt:

$$\begin{matrix} C_2H_5 \\ C_2H_5 \end{matrix}\!\!>\!O + 6\ O_2 \longrightarrow 4\ CO_2 + 5\ H_2O.$$

Ein Drittel der Volumenverminderung entspricht dem Volumen des Äthergases. Das Volumen des bei der Verbrennung entstandenen Kohlendioxyds wird als vier Drittel der Kontraktion berechnet.

Aus der Gleichung geht auch hervor, dass die Ätherverbrennung viel Sauerstoff verbraucht. Soll man z. B. eine Luftprobe mit nur 4 % Äther, z. B. bei einer oberflächlichen Narkose verbrennen, muss das Gasgemisch im Analyseapparat 24 % Sauerstoff enthalten. Bei solchen Analysen ist es also nötig, Sauerstoff zuzusetzen.

Wenn das Gasgemisch mehr als 6 % Äther enthält, geschieht die Verbrennung mit einer Explosion. Bei weniger als 6 % geht sie ruhig von statten.

Bei Verbrennung in dem Haldaneapparat zeigte sich bald, dass der Äther in einer Luftprobe nur selten ganz verschwunden war, ehe man 35 oder 40 Absorptionen ausgeführt hatte. Erst dann wurde ein konstantes Gasvolumen erreicht. Doppelanalysen stimmten ferner mit einander am besten überein, wenn der Platindraht während der Analyse nicht stark erhitzt, sondern nur rotglühend war. Die Übereinstimmung war auch besser, wenn man verhinderte, dass die Verbrennungskammer zu heiss wurde. Darum wurde sie während der Analyse einer Probe mehrmals mit dem Quecksilber im Apparate abgekühlt.

Während der Ausprüfung der hier vorgeschlagenen Methode fand ich bald in Modellversuchen, wo mit Äther versetzte Aussenluft verwendet wurde, dass ein Kohlendioxydunterschuss entstand. Man bekam weniger Kohlendioxyd als die aus der Volumenänderung berechnete Quantität. Es schien wahrscheinlich, dass das für die Hähne gebrauchte Fett damit irgendwie in Zusammenhang stehen könnte. Fett und Äther haben ja starke Affinität zu einander, und es ist auch nicht unwahrscheinlich, dass bei der Erhitzung Reaktionen zwischen dem konzentrierten Sauerstoff und dem Fette ablaufen können. Nach verschiedenen Versuchen ein Ersatzmittel zu finden, wurde Orthophosphorsäure »pro Analysi (KAHLBAUM)« mit befriedigendem Resultate geprüft, wie aus Tabelle 1 hervorgeht.

Tabelle 1.

	Aus der Gesamtvolumenänderung berechnetes CO_2-Volumen in Kubikzentimetern	Bei der Analyse gefundenes CO_2-Volumen in Kubikzentimetern	Fehler in Proz.
Mit Fett	3.100	2.896	— 6.6
	1.793	1.620	— 9.6
	2.436	2.227	— 8.6
	1.309	1.258	— 3.9
	1.083	1.015	— 6.3
Mit o-Phosphorsäure	1.187	1.184	— 0.3
	2.055	2.050	— 0.2
	0.850	0.852	+ 0.3

Die Orthophosphorsäure ist aber so dünnflüssig, dass man öfter als sonst prüfen muss, dass die Hähne dicht halten.

Wie sich die o-Phosphorsäure gegen Äther verhält, wurde nach Füllung eines Absorptionsgefässes des Apparates damit untersucht.

Eine Luftprobe, die 5.67 % Äther enthielt, verlor in der Säure 4.3 % ihres Gesamtvolumens, und konstantes Volumen wurde schon nach 20 Absorptionen erreicht. Atmosphärische Luft dagegen zeigte keine messbare Volumenänderung. Eine Luftprobe mit 20 % Kohlendioxyd verlor etwa 0.5 % ihres Volumens in der o-Phosphorsäure. — Die Tabelle 1. scheint indessen zu zeigen, dass die Affinität zwischen Äther und Phosphorsäure bei den während der Analyse vorhandenen Verhältnissen vernachlässigt werden kann. Die Säure bedeckt nicht die Innenseiten der Röhrchen im Apparate wie es Fett gewöhnlich tut, und darum kommen wahrscheinlich Äther und Säure während der Analyse kaum mit einander in Berührung.

Die Analysemethode ist folgendermassen ausgeformt worden.

Am liebsten benutzt man einen Haldaneapparat, dessen Bürette von 10.00 bis 5.00 (nicht nur bis 6.00) Kubikzentimetern feingraduiert ist.

Die Hähne des Apparates werden mit Orthophosphorsäure gedichtet.

Etwa fünf Kubikzentimeter der Luftprobe werden in den Apparat eingeführt und nach Ablesen des Volumens wird reiner Sauerstoff z. B. aus einer Fussballblase durch das Seitenrohr des Manometers zugesetzt bis die Bürette fast gefüllt ist. Nach erneutem Ablesen führt man das Gas in die Verbrennungskammer ein, der Glühstrom wird geschlossen und so reguliert, dass der Draht schwach glüht. Nach zehn »Absorptionen« wird der Strom ausgeschaltet, und die Verbrennungskammer mit dem Quecksilber gekühlt. Dann wieder Glühstrom, Verbrennung und Abkühlung, so fährt man fort bis die Verbrennung fertig ist, wozu 35 bis 40 »Absorptionen« nötig sind. Dann wird das Volumen abgelesen, und man schreitet zur Kohlendioxydanalyse in gewöhnlicher Weise.

Enthält die Luftprobe mehr als 12 % Äther, soll man weniger als 5 Kubikzentimeter in den Apparat einführen, um Explosion während der Analyse zu vermeiden, stattdessen aber eine grössere Sauerstoffmenge.

Die Methode verlangt, dass man nach jeder achten bis zehnten Analyse eine Kontrollanalyse mit ätherhaltiger Aussenluft macht, um zu prüfen dass die Kohlendioxydmenge noch der Volumenänderung entspricht. Es hat sich nämlich herausgestellt, dass man nach einer wechselnden, aber gewöhnlich geringen Anzahl von Analysen weniger Kohlensäure als berechnet bekommt. Wenn man dabei den Platindraht vertauscht, verschwindet der Fehler sofort.

Es ist also gezeigt worden, dass Kohlensäureanalyse mit den üblichen Absorptionsmethoden irrtümliche Ziffern gibt, wenn

Äther in der Luftprobe ist, wie z. B. bei Narkosen. Die Grösse des Fehlers wechselte, was wahrscheinlich mit dem Sättigungsgrad der Lauge mit Äther im Zusammenhange steht. Hier wird zum Vergleich der Methoden ein Versuch mitgeteilt. Es sind Alveolarluftproben untersucht worden, die von einer Katze in Ätharnarkose nach der von v. EULER und LILJESTRAND benutzten Methode gewonnen sind. Aus der Probepipette wurde die eine Hälfte mit der hier vorgeschlagenen Methode analysiert und die andere in einem anderen Analyseapparate gleichzeitig untersucht, ohne dass der Äther verbrannt wurde.

Tabelle 2.

	Äthergehalt in Proz.	CO_2-Gehalt in Proz. nach Ätherverbrennung	CO_2-Gehalt in Proz. ohne Ätherverbrennung	Fehler in Proz.
Probe 1.	8.02	6.35	6.61	+ 4
Probe 2.	4.67	8.43	9.54	+ 13
Probe 3.	3.36	5.46	5.65	+ 3½

Es muss zugegeben werden, dass die hier vorgeschlagene Methode mit ihren Kontrollen, Drahtvertauschen u. a. etwas umständlich ist. Sie scheint aber jetzt die einzige gasvolumetrische Methode zu sein, die eine sichere Kohlendioxydbestimmung in einer ätherhaltigen Luftprobe erlaubt und mit der es ausserdem möglich ist sowohl Äther als Kohlensäure in derselben Probe zu bestimmen.

Zusammenfassung.

Es wird eine gasvolumetrische Methode beschrieben, die es ermöglicht, Äther und Kohlendioxyd bei gleichzeitigem Vorkommen in einer Luftprobe, z. B. bei Narkosen, quantitativ zu bestimmen. Es ist auch gezeigt worden, dass bei der üblichen Kohlendioxydabsorptionsanalyse mit Kalilauge zu hohe Werte erhalten werden, wenn Äther in der Luftprobe vorhanden ist.

Literatur.

v. EULER, U. S. und G. LILJESTRAND, Skand. Arch. Physiol. 1936. *74*. 101.
GRAMÉN, K., Acta Chir. Scand. 1922. Suppl. I.
KRUSE, T. K., J. Biol. Chem. 1923. *56*. 127.
LILJESTRAND, G. und P. LINDE, Skand. Arch. Physiol. 1930. *60*. 273.

From the Institute for Theoretical Physics, Copenhagen University.

An Apparatus for Automatic Measurement with Improved Accuracy of Weak Radio-Activities by the Geiger-Müller Counter.

By

K. ZERAHN.

Received 13 February 1945.

The use of artificial radioactive elements as indicators in the study of biological problems is rapidly increasing. The initial activities which it is possible to use are often limited e. g. by the danger of thereby introducing a disturbing factor in the experiment, and it is often desirable to measure very weak samples without sacrificing too much of the accuracy.

In this laboratory we use exclusively Geiger-Müller counters, mainly in the form described by HILDE LEVI (1941) with several improvements which are not, however, essential for the arrangement here described. The accuracy with which a fairly strong radioactive preparation can be measured is limited by the statistical error on the number of impulses recorded. On N impulses the absolute uncertainty is $\sqrt{N}$ and the relative $\frac{\sqrt{N}}{N} = \frac{1}{\sqrt{N}}$ or in per cent $\frac{100}{\sqrt{N}}$. When 2 500 impulses are recorded the error will be ± 2 %. By a few comparative measurements of the unknown sample against a known standard sample one can make sure that possible variations in the counter cannot seriously increase the error.

On weak samples it becomes increasingly difficult to obtain accurate measurements. It takes a long time to get a sufficient

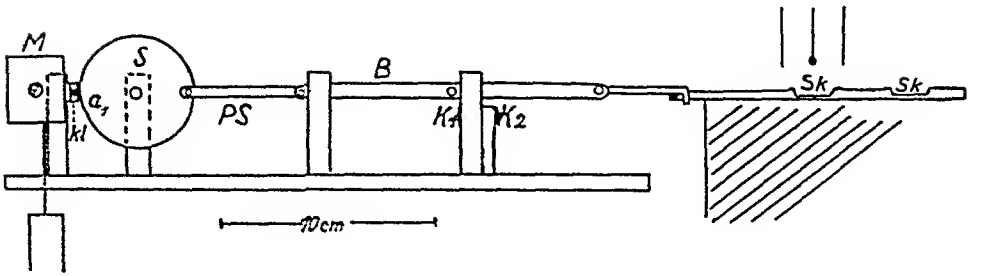


Fig. 1.

number of impulses counted to reduce the statistical error to the point desired, and the influence of the background value of the counter (the zero count — ZC) becomes considerable. This ZC cannot be characterized by the statistical uncertainty alone, because it shows irregular variations, amounting over periods of hours often to more than one imp./min. It is therefore not possible to get accurate results by comparative counts of the zero and the sample each lasting several hours, as they must do to reduce the statistical error. When, however, comparative counts are made over a large number of short periods the influence of the drift of the ZC becomes eliminated. Acting on a proposal from prof. KROGH I therefore undertook to construct an apparatus which can do this automatically.

The principle of the construction is to have the slide carrying the preparation moved back and forth between two positions, placing the preparation exactly under the window of the counting chamber in one and withdrawing it so far in the other that a ZC is made. A clock is started and stopped each time with the preparation and adds up the time t during which the preparation is counted. The change is made by the fastest drum of the counting device each time 100 impulses have been recorded. When we measure the total time T for, say, 5 000 impulses we have recorded 2 500 imp. from the preparation and corresponding to the time t and 2 500 from the ZC corresponding to $T—t$. Each of these counts will have a 2 % statistical uncertainty. Assuming T to be 1 000 min and t to be 400 min, we get for the preparation 6.25 ± 0.12 imp./min and for the ZC 4.17 ± 0.08. The difference, the net count for the preparation is $2.08 \pm \sqrt{0.12^2 + 0.08^2} = 2.08 \pm 0.145$ or an error of ± 7 %, while with long period counts it would be difficult to reduce the uncertainty below 1 imp./min.

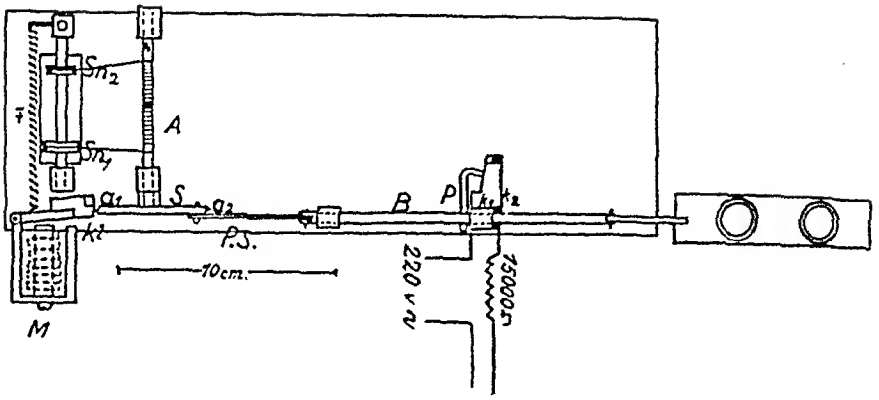


Fig. 2.

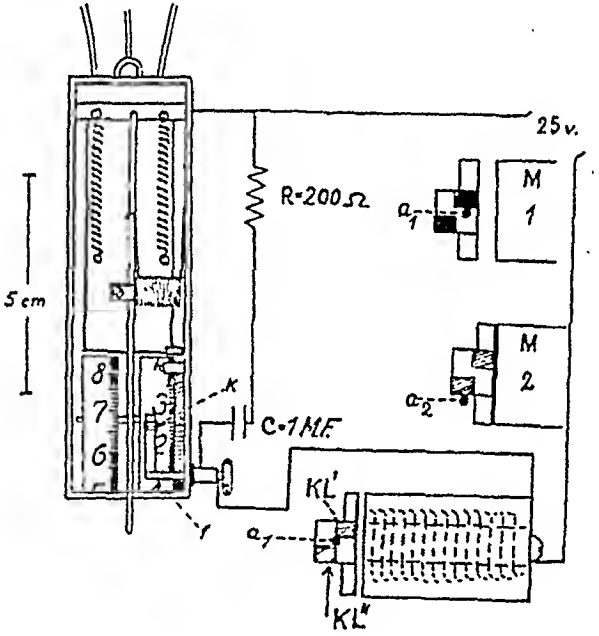


Fig. 3.

Fig. 1 shows the apparatus in elevation, fig. 2 seen from above. Fig. 3 shows the wiring for the magnet and the mechanic counting device. The power to move the slide is obtained from a weight of about $^1/_2$ kg suspended in a steel wire (Sn_1) wound on the shaft (A fig. 2). The fig. shows also a wire (Sn_2) wound in the opposite direction and holding a small weight. The two wires are not wound directly on the shaft, but on a thinwalled tube, fastened to the shaft by a bayonet clutch. When the tube is loosened the wire (Sn_1) can be wound on by pulling (Sn_2) off, so as to rapidly prepare

the apparatus for a fresh set of countings. On the shaft (A) the circular disc (S) is mounted and connected by the rod (PS) to the sliding rod (B) which can be hitched on to the slide of the counter. On the edge of the disc (S) two steel pins (a_1) and (a_2) are arranged in the same diameter with the connecting rod. These will come up against the stop (Kl) mounted on the anchor of a small electromagnet (M). One pin only can pass the stop when the anchor is attracted by the magnet. The action of the stop is illustrated in figs. 1 and 3. The hatched areas in fig. 3 are a few mm higher than the others. The pin (a_1) is pressed upwards by the weight, but is kept in place by the stop. When the anchor is attracted (a_1) will slide off (Kl'), but (a_2) will be stopped by (Kl''). When the anchor is released (a_2) will move from (Kl'') to (Kl') which does not cause any perceptible movement of the slide. A spiral spring (F fig. 2) pulls the anchor back when no current is on. The current is put on by the fast drum of the mechanical counter (fig. 3). On to this is soldered a small wedge (K) which touches a contact spring (f) when the drum records 0 and 1. The spring is isolated from the metal case of the counter. To avoid sparking and electrical disturbances which might affect the counting the condenser ($C = 1 M.F$) and resistance ($R = 200\,\Omega$) are arranged. As a source of current we have a rectified alternating current, but a 25 v. dry battery serves very well. On the sliding rod (B fig. 2) a bent pin (P) presses two contact springs (K_1 and K_2) together when the slide is pushed in. (These must remain in contact while the anchor is being attracted by the magnet.) (K_1) is fixed to the metal stand while (K_2) is isolated. They connect the self starting synchronous clock with the A. C. mains through a 15 000 Ω resistance to reduce the electrical disturbance which might otherwise act on the amplifier system and give rise to spurious impulses.

A control on the working of the apparatus can be obtained by measuring the distance through which the weight has been lowered, since 100 impulses correspond to a definite length of wire.

It is evident that the apparatus cannot conveniently be used for radioactive substances having a half life period less than about 12 to 24 hours, but this does not matter much since the biological applications usually require rather long-lived substances. We have used the apparatus mainly for the determination of weak preparations of phosphorus and sulphur. The preparations are fixed in aluminium dishes and these placed in the slide.

Even with a counter showing normally a constant ZC it will be convenient to use the apparatus, because it provides a control of the ZC which is extremely useful because the counter may become soiled from traces of active preparations. Soiling of the counter itself will raise the ZC, but not otherwise give trouble, but soiling of the slide may be troublesome and is discovered by taking two ZC simultaneously, which should not differ by more than the statistical uncertainty. For this purpose the slide is supplied with 2 indentures for samples. It is possible also to measure two weak preparations one against the other, but for this purpose other arrangements are preferable.

Summary.

An apparatus is described purporting to increase the accuracy in measurements of weak radioactivities by the Geiger-Müller counter. This is important because the background count often varies a great deal more than can be accounted for by the statistical uncertainty. To avoid the effect of this variation preparation and zero count are measured alternately at short intervals. This is performed automatically by the apparatus described.

Reference.

Levi, Hilde, Acta Physiol. Scand. 1941. *2*. 311.

Biological Institute, Carlsberg Foundation, Copenhagen.

Cellulose Sulfuric Acids as Anticoagulants.

By

TAGE ASTRUP and JØRGEN PIPER.

Received 13 February 1945.

In continuation of our investigations on synthetic polysaccharide sulfuric acid esters as anticoagulants in blood clotting (ASTRUP, GALSMAR and VOLKERT (1944)) we have studied the sulfuric acid ester of cellulose glycollic acid prepared by KARRER, KOENIG and USTERI (1943). This substance was said to be sufficiently non-toxic to be used for clinical purposes.

In previous investigations (ASTRUP, GALSMAR and VOLKERT (1944)) it was found that cellulose sulfuric acid esters prepared in the usual manner could be precipitated by addition of concentrated salt solutions, e. g. of NaCl. We have now found that, when the heating of the reaction mixture is continued for several hours or at a higher temperature, products *soluble* in concentrated salt solutions are obtained. By dissolving in water the substances prepared in this manner, solutions of low viscosity were obtained, while the earlier products gave solutions of high viscosity. The anticoagulant activity was only moderately decreased, while the new products were considerably less toxic than the old cellulose sulfuric acid esters; but just as the glycollic acid derivatives, they were not found completely non-toxic.

A. Preparation of the Substances.

Cellulose Glycollic Acid: This substance was prepared (compare CHOWDHURY (1924)) by treating 5 g of cellulose in 3 hours with 40 ml. of a 40 per cent NaOH-solution and then adding 20 g of monochloroacetic acid in small portions. After standing for half an hour another 40 ml. of the NaOH-solution and 20 g of chloroacetic acid was added and the mixture left standing to the next day. The mixture was then treated

with 96 per cent ethyl alcohol (about 100 ml.) and dissolved again in water (about 100 ml.) and this procedure was repeated three times. The solution was then centrifuged for one hour in order completely to remove any cellulose left unchanged. The clear solution was now re-precipitated with alcohol several times (with addition of a few ml. of a saturated NaCl-solution) until neutral reaction. After treatment with alcohol it was dried in the air. Yield 5 g of the sodium salt. (K—96).

Sulfuric Acid Ester of Cellulose Glycollic Acid: 2.5 g of cellulose glycollic acid (as sodium salt) is heated to 115—120° for one hour (oil bath) in a mixture of 7.5 ml. of chlorosulfonic acid in 50 ml. of α-picoline. After cooling it is suspended in 200 ml. of water and centrifuged. The solution is neutralized with 2-n NaOH and precipitated with 600 ml. (2 vol.) of ethyl alcohol. The precipitate is dissolved in 100 ml. of water and dialyzed against tap water. After filtering it is concentrated in vacuo to about 90 ml., a few ml. of saturated NaCl-solution added and precipitated with 1.5 vol. of ethyl alcohol. The precipitate is isolated and dried with alcohol. Yield 1.4 g. (K—107.2).

KARRER (l. c.) used a considerably lower temperature (85°), but we obtained only good yields at higher temperatures, both with pyridine and α-picoline as reaction medium.

Cellulose Sulfuric Acid Soluble in Concentrated Salt Solution: A mixture of 50 ml. of α-picoline, 7.5 ml. of chlorosulfonic acid and 2.5 g of cellulose is heated for one hour to 115—120° in an oil bath. After cooling it is poured into 200 ml. of cold water. 100 ml. of 2-n NaOH is added and the solution is precipitated by addition of 300 ml. of saturated sodium chloride solution. After centrifuging the precipitate is dissolved in 50 ml. of water, again centrifuged and then precipitated with one vol. of alcohol. After treatment with alcohol and drying in the air it weighs 3.1 g. (K—106.1.)

This product constitutes the cellulose sulfuric acid which is *insoluble* in concentrated salt solutions. The *soluble* substance is found in the supernatant liquid from the centrifuging, which is now precipitated by addition of one vol. (600 ml.) of alcohol. After centrifuging the precipitate is dissolved in 100 ml. of water, dialyzed against running tap water for two days and concentrated in vacuo to 50 ml. After addition of a small amount of saturated NaCl-solution it is precipitated with 75 ml. of alcohol. It is filtered, treated with alcohol and dried in the air. Yield 3.0 g. (K—106.2).

By changing the composition of the reaction mixture, the reaction time or the temperature, it was found possible to increase the yield of the soluble substances, and in some cases only traces of the insoluble products were left. Other polysaccharides were treated in a similar manner and yielded also products of lower toxicity, see later under Section C.

B. Properties of the Substances.

The properties of the substances thus prepared were investigated according to methods previously described, the anticoagulant

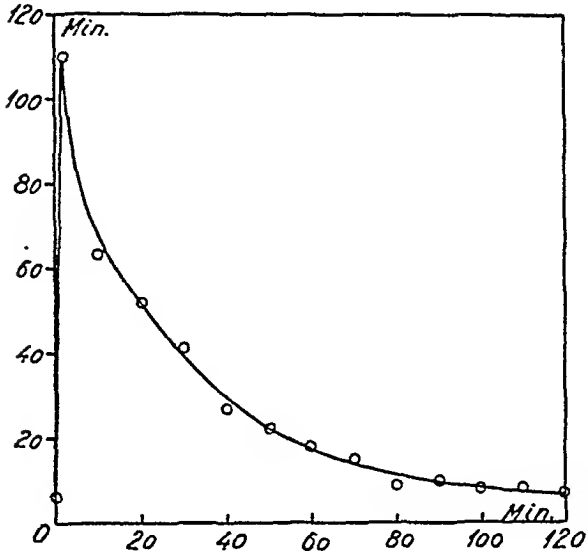


Fig 1.

The action of the sulfuric acid ester of cellulose glycollic acid on the clotting of blood in a rabbit. *Abscissa:* Time (in minutes) after the intravenous administration of 2 mg. per kg. of the substance. *Ordinate:* Clotting time (in minutes) of blood removed from a carotid.

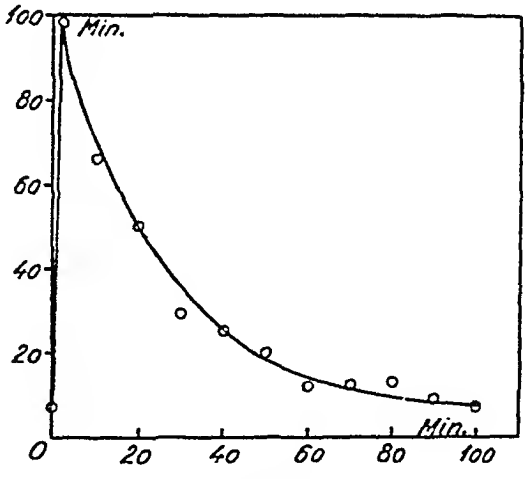


Fig 2.

A similar experiment as in Fig. 1 using 2 mg. per kg. of a soluble cellulose sulfuric acid (K—84.2).

activity preferably according to the method described by ASTRUP, GALSMAR and VOLKERT (1944) and the toxicity according to the platelet method, PIPER (1945 a).

The anticoagulant activities of the sulfuric acid ester of cellulose glycollic acid and that of the soluble cellulose sulfuric acid are of the same order, about a third to one half of the potency of a cellulose trisulfuric acid (C—12) prepared as usual (ASTRUP, GALSMAR and VOLKERT (1944)) and used as a comparison sample.

In Table I are shown some typical results of the experiments on intravenous administration of the substances on rabbits. The number of platelets are given in thousands.

Table I.

Substance	Dosis mg per kg	Number of Platelets		
		before	after	
Cellulose glycollic acid	3 mg	912	871	No clusters
(K—71.1; K—73.1)	5 »	670	645	» »
Sulfuric acid ester of cellulose glycollic acid (K—81.1; K—107.2)	1 mg	650	652	No clusters
	5 »	620	530	» »
	10 »	672	482	Single clusters +
Insoluble cellulose sulfuric acid (K—84.1; K—106.1)	2 mg	990	400	Single clusters +
	5 »	416	86	Clusters ++
	10 »	811	440	Clusters ++
Soluble cellulose sulfuric acid (K—84.2; K—106.2)	5 mg	687	684	No clusters
	10 »	760	578	A few clusters.

The action on the blood coagulation *in vitro* of the substances prepared is very similar to that of heparin, and shown in Fig. 1 and Fig. 2.

C. Discussion.

The new substances are considerably less toxic than the substances previously prepared, as they agglutinate the thrombocytes only to a very moderate degree. Especially in the doses used for practical purposes they may be assumed to be almost harmless. They may be used for experimental purposes, but are not sufficiently non-toxic to be used for therapeutic purposes, as in this case very rigid claims of harmlessness must be made. In his paper KARRER (l. c.) writes: "Die Toxicitätsbestimmungen der Polysaccharid-polyschwefelsäure-ester streuen sehr erheblich; offenbar

spielen individuelle Unterschiede der Tiere bei der Verträglichkeit der Präparate eine grosse Rolle. Um ein zuverlässiges Urteil über die Giftigkeit dieser Substanzen zu gewinnen, sind daher sehr zahlreiche Toxicitätsbestimmungen unerlässlich."

According to our opinion it is not sufficient for the substances here in question to determine a mean value of the toxicity. In our experiments with polysaccharide polysulfuric acids we have often obtained grave symptoms by intravenous administration of doses which in other animals gave no signs of toxicity, neither macroscopically nor microscopically. The reason for this great individuality may lie in the mode of action of the substances, as their toxic properties are due to the agglutination of the thrombocytes. The decrease in the number of thrombocytes may vary considerably from animal to animal, using the same dose of the same preparation. It is therefore impossible with safety to predict the severity of the platelet agglutination by administration of a dose to an animal, and even if only a few per cent of the animals used respond with any signs of severe intoxication, this must be sufficient to preclude the use of such a substance in man.

If therefore a substance of this kind is not found to be completely harmless in all cases and in doses considerably higher than those which may be used in practice, it must necessarily be called a toxic substance. According to KARRER (l. c.) clinical investigations with his substances are under way. We do not, however, hold any of the substances investigated by us safe enough to warrant such a test. A sample of cellulose glycollic acid sulfuric acid ester, kindly furnished by Professor, Dr. PAUL KARRER, Zürich, was compared with our products and showed similar properties.

It is interesting that it is possible only by changing the reaction conditions to obtain cellulose sulfuric acids which are almost non-toxic, while the anticoagulant potency is only moderately decreased. A splitting of the cellulose molecule may be assumed, yielding degradation products of lower molecular weight, but still of a colloidal nature and therefore retaining most of the inhibitory action.

By treating rice and potato starch in the same manner (5 g. to 7.5 ml. chlorosulfonic acid in 50 ml. of α-picoline, one hour at 115—120° C.) similar products are obtained. The anticoagulant strength is about one half of the strength of the cellulose trisulfuric acid (C—12) used as comparison sample, and they also are less

toxic (10 mg. per kg.: blood platelets: 505 → 264 (rice), 608 → 125 (potato)). The sulfuric acid esters of starch prepared in the usual manner were very toxic substances.

Methyl cellulose yields a substance with a potency of the same order but with a considerably higher toxicity (10 mg. per kg.: blood platelets: 600 → 51). Acetyl cellulose gives under the same conditions only small amounts of an inactive, non-toxic product. Almost all the acetyl substance is (especially at 100°) left as a substance insoluble in cold water. By heating it is dissolved and after cooling the solution is transformed into a comparatively rigid gel. Further investigations of the substances here mentioned are described in connection with a comprehensive study of the properties of synthetic polysaccharide sulfuric acids, PIPER (1945 b).

This work was aided by grants from *"Danmarks tekniske Højskoles Fond for teknisk Kemi"* and from *"Lovens kemiske Fabriks Legat til Minde om Apoteker August Kongsted"*.

Summary.

The preparation and properties of a sulfuric acid ester of cellulose glycollic acid and of a cellulose sulfuric acid ester soluble in concentrated salt solutions are investigated.

The toxicity of the substances prepared are decreased considerably in comparison with the toxicity of the synthetic polysaccharide sulfuric acid esters previously investigated, while the anticoagulant activity is only moderately decreased.

Literature.

ASTRUP, T., I. GALSMAR and M. VOLKERT, Acta Physiol. Scand. 1944. *8*. 215.

CHOWDHURY, J. K., Biochem. Z. 1924. *148*. 76.

KARRER, P., H. KOENIG and E. USTERI, Helv. Chim. Acta 1943. *26*. 1296.

PIPER, J., Acta Physiol. Scand. 1945 a. *9*. 28.

PIPER, J., Farmakologiske Undersøgelser over syntetiske heparinlignende Stoffer. (Disp.) 1945 b. Copenhagen.

From the Biological Institute of the Carlsberg Foundation, Copenhagen.

The Influence of Ionic Strength on the Acid Liver Phosphatase.

By

SØREN LØVSTRUP JENSEN.

Received 26 February 1945.

It was shown by ASTRUP (1942, 1944) that the activity of thrombin is greatly influenced both by pH-displacements and by variations in the ionic strength of the solutions. In the last-mentioned paper it is supposed, with reference to an investigation by BAMANN and SALZER (1936 a), that also the action of the acid liver phosphatase may depend on the ionic strength of the medium. BAMANN and SALZER show that by using citrate buffer prepared according to SØRENSEN two maxima of activity are obtained (at pH 4 and 5.5). If the concentration of citrate is kept constant, only one maximum at pH 5.5 is found.

LUNDSTEEN (1938), however, has shown that the presence of citrate makes the colorimetric determination of phosphorus uncertain, as the extinction, for the same amount of phosphorus, decreases with increasing amounts of citrate, while acetate is without disturbing influence on the determination. The minimum of activity evident from the curves found by BAMANN and SALZER corresponds exactly to the pH-value, where the concentration of citrate is the largest, when the buffers are made as described by SØRENSEN. The deflection of the pH-activity curve found by these authors is therefore in all probability only a result of the presence of citrate and has nothing to do with the action of the phosphatase. Hence it is not possible to draw any conclusions based on the results of BAMANN and SALZER, and the purpose of the present investigation is, with the use of acetate buffers, to disclose any influence which the ionic strength might have on the action of the acid liver phosphatase.

The enzyme is prepared from thoroughly ground ox liver treated 3 times with acetone and once with ether and dried at room temperature. The extraction is carried out with a twenty-fold volume of n/40 acetic acid, cf. BAMANN and SALZER (1936, b). The enzyme solution is kept at 0°.

Acetate buffer is used in all experiments. For some experiments the acetate buffer is prepared in the usual manner by changing simultaneously the contents of sodium acetate and of acetic acid. Other experiments have been made with a buffer solution containing a constant amount of sodium acetate and varying contents of hydrochloric acid, by which means the ionic strength is kept constant.

A 0.25 molar solution of β-glycerophosphate (sodium salt) is used as substrate, and the pH value of the resulting solutions is determined with a glass electrode.

To the experiments 4.0 ml of the enzyme solution and 1.0 ml of the substrate are used. Buffer, salt solution and water are added until the total volume is 20 ml. Then one drop of toluene is added and the samples are placed for 24 hours at 34°. In 5 ml of the mixture the proteins are precipitated by the addition of 5 ml of 10 per cent trichloro-acetic acid. After centrifuging, the free phosphate in 5 ml of the centrifugate is determined according to FISKE and SUBBAROW (1925), using a Pulfrich photometer, and the total amount of free phosphorus in the sample is calculated. The results are shown in the graphs.

Fig. 1 shows that the addition of a constant amount of sodium chloride to the reacting solution (with acetate buffer of the usual composition) does not change the position of the pH-optimum for the reaction (about pH 5).

In Fig. 2 an acetate buffer of the usual composition is compared with an acetate buffer of constant ionic strength. The activity of the enzyme decreases at the lower pH values, when the ionic strength is kept constant ($\mu = 0.1$). At the higher values the ionic strength of the two buffer solutions used is about the same, and the enzyme activities of the two solutions are approaching each other. The pH-optimum is a little higher than in fig. 1, possibly due to the circumstance that another (older) enzyme solution is used.

Fig. 3 shows the results of the addition of different salts in varying concentrations, pH being kept at 4.8.

Previous investigations on the action of electrolytes on phosphatases have, as a rule, aimed at the finding of specific inhibitors or activators for the enzyme reaction in question, and the salts have therefore been used in small concentrations. Thus INOUYE (1929) found that fluoride, sulfate and oxalate inhibit the action of glycerophosphatases from Aspergillus oryzae, BAMANN and

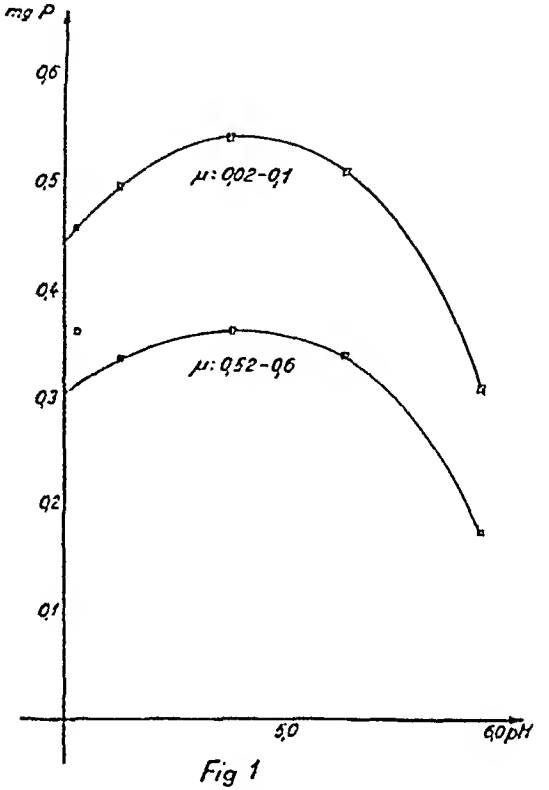


Fig. 1. Relation between pH and action of the acid liver phosphatase with and without the addition of sodium chloride.

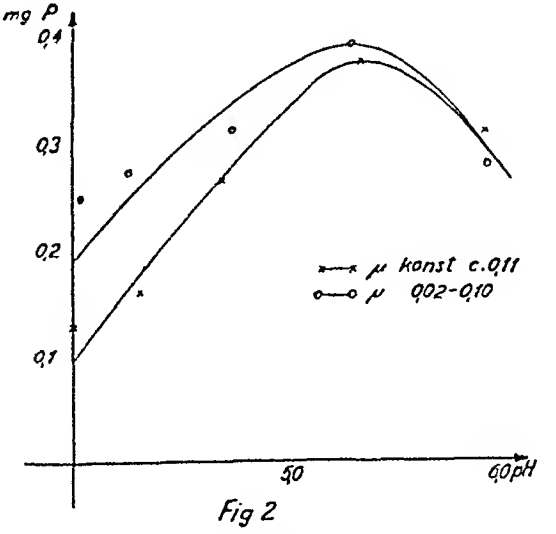


Fig. 2. Relation between pH and action in acetate buffer at constant or varying ionic strength.

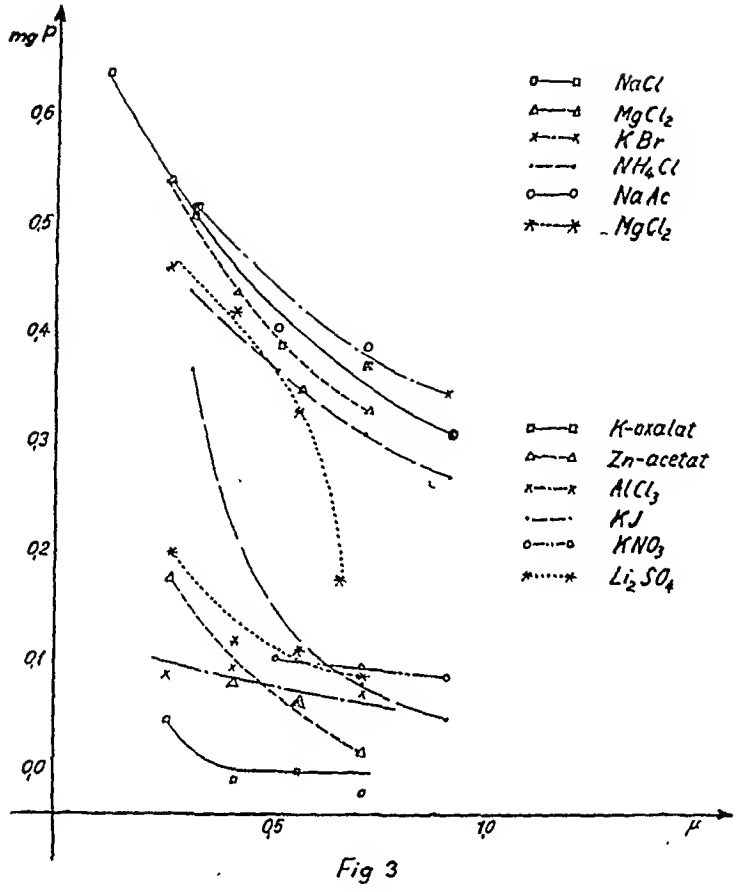


Fig. 3. The inhibitory action of different salts in relation to the ionic strength.

RIEDEL (1934) showed that the Mg^{++}, contrary to its action on the alkaline phosphatase, is unable to activate acid phosphatases of animal origin. In higher concentration it acts as an inhibitor. MORII (1933) has shown that the chlorides of Cu, Fe and Mn prevent the phosphatase action. BELFANTI and collaborators (1935) have found that the action of the acid liver phosphatase is retarded by oxalate and fluoride, while the alkaline phosphatase is not influenced.

From the results here published (Fig. 3) it is seen that the salts investigated may be divided into two groups. The first group includes potassium oxalate, potassium nitrate, lithium sulfate, zink acetate, aluminium chloride and, to some degree, potassium iodide. It may be assumed that these substances exhibit a specific inhibitory influence on the enzyme system, even if the mode of action probably is not the same for the different salts. The second

group, containing sodium chloride, magnesium chloride, potassium bromide, ammonium bromide, sodium acetate and probably mangano chloride, shows an inhibitory action, which, with reasonable accuracy, seems dependent on the ionic strength of the solution. It may be assumed that this inhibitory effect is due to a salt effect. Potassium iodide and mangano chloride are intermediate between the two groups; in small concentrations they do not exhibit any specific action.

Summary.

It is found that some electrolytes exert an unspecific inhibitory action on the activity of the acid liver phosphatase. This inhibitory action seems to depend on the ionic strength of the solution. Other salts, however, exhibit a specific inhibitory action.

This investigation was made at the suggestion of Dr. TAGE ASTRUP, to whom I wish to express my best thanks for advice during the work.

References.

ASTRUP, T., Biochem. Z. 1942. *313*. 229.
ASTRUP, T., Acta Physiol. Scand. 1944. *7*. Suppl. XXI.
BAMANN, E., and E. RIEDEL, Hoppe-Seyl. Z. 1934. *229*. 125.
BAMANN, E., and W. SALZER, Biochem. Z. 1936 a. *280*. 143.
BAMANN, E., and W. SALZER, Ibidem, 1936 b, *280*. 147.
BELFANTI, S., A. CONTARDI, and A. ERCOLI, Biochem. J. 1925. *29*. 517.
FISKE, C. H., and Y. SUBBAROW, J. Biol. Chem. 1925. *66*. 357.
INOUYE, K., J. Biochem. 1929. *10*. 395.
LUNDSTEEN, E., Enzymologia, 1938. *5*. 383.
MORII, I., cited after Ber. ges. Physiol. 1933. *72*. 725.

From the Medical Clinic of the Serafimer Hospital and the Physiological Laboratory of Karolinska Institutet, Stockholm.

The Effect of Some Drugs on the Chemoceptive Fibre Activity in the Carotid Sinus Nerve.

By

B. GERNANDT and Y. ZOTTERMAN.

Received 9 March 1945.

From previous investigations (EULER, LILJESTRAND and ZOTTERMAN 1939 a, 1939 b, 1941) it has been demonstrated that lobeline and nicotine most likely exert their stimulating action upon peripheral neural junctions in the chemoceptive fibres of the carotid body. The object of the present experiments was to investigate whether the strong stimulating effect upon the circulation and the respiration exerted by some related drugs is due at least to one part to a direct excitation effect upon the chemoceptive fibres of the nerve of Hering.

Technique and Procedure.

The experiments have been performed on cats anaesthetized by intravenous injection of 0.05 g chloralose per kg body weight. In order to obtain good space for placing the electrodes the carotid sinus region is widely exposed by resecting the upper part of the muse. sterno-cleidomastoideus and removing the large lymph gland which covers the carotid sinus. The glossopharyngeal nerve is easily found as it passes closely along the tympanic bulla. After resecting the lateral part of the digastric muscle the glossopharyngeal nerve is cut as centrally as possible. By holding the central end of the nerve the branch sent off to the carotid sinus can now be dissected free. In order to obtain as high a signal-to-noise ratio as possible the common sheath of the nerve is pealed off. This is best effected by pinching the sheath at the central cut end of the glossopharyngeal nerve and by drawing it off over the nerve as a sock. In this way the sheath comes off from the tiny carotid sinus nerve without any undue stretching of the nerve fibres.

A cannula is introdueed in the central part of the lingual artery. After elamping the external carotid artery, this cannula is used for introdueing the agents to be tested directly into the sinus region. The arterial blood pressure is recorded from the femoral artery by a Hg-manometer and the respiratory movements by means of a simple Marey pneumograph. The actions potentials from the nerve of Hering were studied by means of an amplifier and a cathode ray tube previously deseribed (ZOTTERMAN 1939).

The injeetion of ordinary Ringer solution into the carotid sinus is normally followed by a deerease in the frequeney of small aetion potentials from the nerve. This effeet is due to the biearbonate content of the solution and this must be borne in mind when judging the effect of various drugs administrated in Ringer solution. Intra-arterial injeetion of lobeline 1 : 100,000 in Ringer solution gives however an immediate very strong excitatory effect on the nerve as has been recorded previously (EULER, LILJESTRAND and ZOTTERMAN 1939). Lobeline ean thus be used as a test upon the funetional ability of the chemoceptive fibres. When the nerve preparation responds to an intra-arterial injeetion of a few μg of lobeline we may feel sure about that we have chemoceptive fibres in function.

Results.

Nicetamide and pentazol. According to experiments by E. ZUNZ and P. TREMONTI (1931) coramin exerts its stimulating effect upon the respiration by a direct action upon the respiratory centre as well as by a reflex action via the carotid sinus region. By recording the act'on potent'als from Herings' nerve, when injecting appropriate amounts of the drug intra-arterially we could at once get a definite proof of its peripheral action. We have for that reason tested some closely related substances, nicetamide (pyridinoyldiaethlylamide) and pentametylentetrazol in doses from a few μg to 50 mg.

In the larger doses these substances when injected intra-arterially elicited a very marked rise in the arterial blood pressure and an apnoea of short duration very similar to the effect of adrenaline in larger doses. Injections of amounts less than 1 mg. had no effect upon the blood pressure. No effect whatever could however be observed upon the activity of the chemoceptive fibres. We have repeated these experiments with the same negative results in altogether six cats in which the nerve preparation always responded promptly to a few μg of lobeline. It thus seems obvious that the stimulating effect of these drugs upon the respiration must be elicited entirely centrally.

Piperidine. U. S. v. EULER (1944) has recently shown that piperidine is excreted normally with the urine and that the excretion is very considerably augmented during muscular exercise.[1] Its typical nicotine like pressor action would suggest that it might stimulate the chemoceptive fibres of the glomus caroticum. This suggestion was proved to be true. It was found that piperidine was highly effective upon the carotid body. Thus as small a dosis as 1.5 μg in 0.2 ml. Ringer solution injected intra-arterially elicited a very definite response. The effect of piperidine is shown in fig. 1, C and D, where its action is compared by that of lobeline (Fig. 1 B). As the sinus nerve is cut, the intra-arterial injection on this side of these small quantities has no effect on respiration or blood pressure.

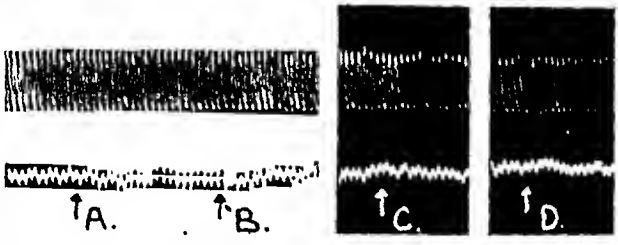


Fig. 1 a. Cat 3.1 kg, chloralose anaesthesia. Upper curve respiratory movements, lower curve arterial blood pressure. The cat is breathing air spontaneously. The arrows mark the intra-arterial injection of B) 1.5 μg lobeline, C) 1.5 μg piperidine and D) 15 μg piperidine. The arrow A shows the moment when the electro-neurogram A of fig. 1 b is taken. Blood pressure 130 mm. Hg.

It will be found, that the action of piperidine upon the carotid body seems to be of very much the same strength as that of lobeline. There is however one difference between these two substances, i. e. the action of lobeline persists longer than the action of piperidine. This may be due to differences in diffusion rates, as the latter substance has a lower molecular weight and it might thus be eliminated more quickly from the seat of its action.

These facts raise the question whether piperidine physiologically might exert a regulating function upon the circulation and the respiration. The extreme sensitivity of the carotid body to piperidine demonstrated in this research seems to us to speak in favour for such a view. It may not be impossible that the increased liberation of piperidine during muscular exercise as discovered by

[1] Lecture given to Svenska Läkarsällskapet 20 Febr. 1945.

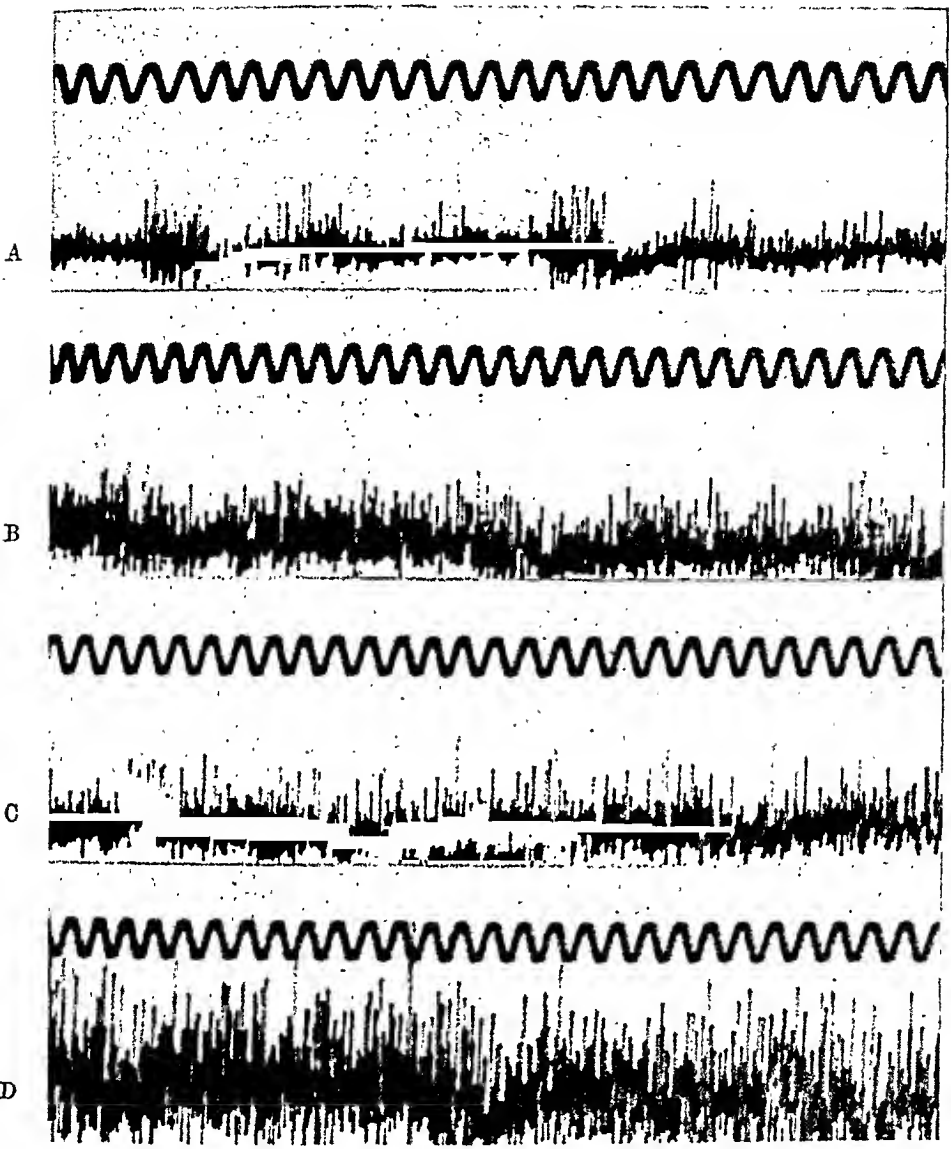


Fig. 1 b. Action potentials from the carotid sinus nerve of cat (3.1 kg.) when breathing air spontaneously. Blood pressure 130 mm. Hg. *A*. Control. *B*. After an intra-arterial injection of 1.5 μg lobeline in 0.2 ml Ringer solution. *C*. After an intra-arterial injection of 1.5 μg piperidine in 0.2 ml Ringer solution. *D*. The effect of 15 μg piperidine intra-arterially. Cf. fig. 1 a. Time 1/50 sec.

U. S. v. EULER, may render a concentration of this substance in the blood sufficient enough to exert an action upon the respiratory functions.

Summary.

1. The effect of nicetamide, pentametylentetrazol and piperidine introduced directly into the carotid sinus has been tested by recording the action potentials from Hering's nerve.

2. Nicetamide and pentametylentetrazol do not exert any direct action upon the afferent mechanisms of the carotid sinus region. Their stimulating effect upon respiration is thus of entirely central origin.

3. Piperidine exerts a very strong excitatory action upon Hering's nerve, the strength of which is very similar to that of lobeline.

4. The physiological significance of this effect of piperidine is briefly discussed in relation to the recent work by U. S. v. Euler.

References.

Euler, U. S. v., Acta Physiol. Scand., 1944. *7*. 285.
Euler, U. S. v., Ibidem. 1944. *8*. 380.
Euler, U. S. v., G. Liljestrand and Y. Zotterman, Skand. Arch. Physiol. 1939. *83*. 132.
Euler, U. S. v., G. Liljestrand and Y. Zotterman, Uppsala Läkarför. Förhandl. 1939. *45*. 373.
Euler, U. S. v., G. Liljestrand and Y. Zotterman, Acta Physiol. Scand., 1941. *1*. 383.
Zotterman, Y., J. Physiol. 1939. *95*. 1.
Zunz, E. and P. Tremonti, Arch. int. Pharmacodyn. 1931. *40*. 449.

From the Departments of Pharmacology and Physiology,
Karolinska Institutet, Stockholm.

Adrenaline Apnoea.

By

B. GERNANDT, G. LILJESTRAND and Y. ZOTTERMAN.

Received 21 March 1945.

If a suitable amount of adrenaline is injected intravenously, the following rise in blood pressure is usually accompanied by a diminution of the depth and rate of respiration which sometimes goes so far that an apnoea results. These phenomena were first described in the rabbit and the dog by OLIVER and SCHÄFER (1895) and have since been demonstrated also in the cat. ROBERTS (1921) found that a similar effect was observed if the blood-pressure was raised quickly by compression of the abdominal aorta. By a process of exclusion he came to the opinion that adrenaline produces its effect upon respiration by direct action upon the medulla. He assumed that it causes a vasoconstriction of the vessels of the medulla, so that the centre is paralysed by want of oxygen or by an accumulation of metabolites. The same view was held by MELLANBY and HUGGETT (1923). Obviously the explanation does not apply to the apnoea after aorta compression. At that time, however, the rôle of the sinus mechanism for the respiratory function was unknown, and with this new knowledge it became necessary to reinvestigate the matter. Experiments on dogs and cats performed by HEYMANS and BOUCKAERT (1930) and by WRIGHT (1930) proved that the adrenaline apnoea is mainly of reflex origin. If both vagi were cut and the carotid sinuses denervated, the action was practically abolished, only a very small effect remaining. According to HEYMANS and BOUCKAERT, the adrenaline apnoea is due to a reflex inhibition of the respiratory centre, produced by the increase of the blood-pressure in the cardio-aortic and sinus circulatory areas. This explanation is in

harmony with the view held by these authors that the nerves of the carotid sinus exert an inhibitory respiratory tonus which is maintained by the normal blood-pressure. In support of this assumption, two observations are quoted:

1) Perfusion experiments of the sinus have demonstrated that respiration can become depressed and even arrested, if the perfusion pressure is raised sufficiently, whereas, at a low perfusion pressure, respiration is increased. These effects are abolished by denervation of the sinuses.

2) Clamping of the two common carotids when the sinus nerves are intact is followed by an increase in respiration; denervation renders the clamping ineffective on the respiration.

Serious objections must be raised, however, against an interpretation according to which these observations prove a direct influence from the baroceptors on respiration. Thus, EULER and LILJESTRAND have pointed out (1937) that variations in the blood-pressure may greatly influence the blood-flow through the carotid body and, thereby, the chemical stimulation from the blood. In their perfusion experiments, increased perfusion-pressure sometimes caused a diminution of ventilation or apnoea, especially if the preceding pressure was low, which in itself caused a hyperventilation. This is in favour of the view that the decrease in ventilation is dependent on a diminution or abolition of the chemical stimulation of the carotid body. No evidence was obtained that a direct inhibitory influence on respiration is exercised by the arterial blood-pressure itself on the sinus region. Similar results were obtained by BJURSTEDT and HESSER (1942). In their experiments a special perfusion cannula was used, so that the sinuses were relatively well supplied with oxygenated blood even at low perfusion pressures. Under these conditions, considerable lowering of the perfusion pressure sometimes called forth an increase in ventilation, but this appeared only after a long reaction time and grew slowly, whereas a rather sudden onset was to be expected in case of a reflex from the baroceptors. After the reflexes on the blood pressure had been abolished, it was still possible by raising the perfusion-pressure to obtain decreased ventilation. BJURSTEDT and EULER (1942) also came to the conclusion that, under normal conditions, respiration was not reflexly influenced from the baroceptors of the sinus and aorta regions, though they observed such an influence after section of the vago-depressor nerves.

With regard to the effects on respiration of clamping the common carotids, SAMAAN and STELLA (1935) interpreted these as the result of variations in the oxygen supply, and EULER and LILJESTRAND (1936) added some new evidence in support of their view. RUDBERG (1938) could demonstrate that the stimulating effect on respiration of clamping the carotids in the rabbit or the cat was enhanced by lowering the blood-pressure below a critical value. Later on (1940), he found that this effect could be counteracted to a certain extent by inhalation of oxygen. WISING (1937) observed a decrease in the alveolar carbon-dioxide tension in anemic patients but not in cases of essential hypertension, so that the assumption that the endosinusal pressure per se would elicit a reflex effect on respiration received no support. All the evidence available makes it very probable that the effects on respiration of clamping the carotids are due, not to the abolition of an inhibition but to a stimulation of chemoreceptors. A direct demonstration that such a stimulation is evoked by the clamping was given by EULER, LILJESTRAND and ZOTTERMAN (1939), who found a considerable increase in the discharge from the chemoreceptors in the sinus nerve when the carotids were clamped.

In the light of the investigations quoted, it seemed very doubtful whether the adrenaline apnoea is to be attributed to a direct inhibitory influence from the baroceptors of the sinus and aorta regions. It would seem to be in better harmony with the known facts to assume that the increased arterial pressure will provide a better blood-flow through the carotid and aortic bodies and thereby diminish the stimulation of the chemoreceptors in those regions. It is of interest to mention in this connection that BORUTTAU (1899) found that the change in respiration was produced when adrenaline raised an abnormally low blood-pressure to a normal height, the respiratory effect thus not being due to an abnormally high blood-pressure.

Methods.

All our experiments have been performed on cats in chloralose anesthesia, 0.06 g per kg body-weight being injected intravenously. The respiration has been recorded partly by means of the body plethysmograph previously described by EULER and LILJESTRAND (1936), and partly by the aid of a simple pneumograph in connection with a Marey tambour. The latter method had to be employed when the ac-

tion potentials were simultaneously recorded. The arterial blood-pressure was recorded from the femoral artery by means of a Hg-manometer. Intravenous injections were made through the femoral vein. The impulse traffic in the carotid sinus nerve was studied by recording the action potentials by means of an amplifier and cathode-ray oscillograph formerly described (ZOTTERMAN 1936). The cats either respired air spontaneously or were given to breathe pure oxygen or gas mixtures from a small Krogh spirometer via Müller valves.

Results.

I. Variations in Oxygen and Carbon Dioxide Tension.

If the decrease in respiration after adrenaline is due to a richer blood supply to the carotid and aortic bodies with a corresponding lessening of the local oxygen want or carbon dioxide accumulation then it is to be expected that the effect should be modified if the tensions of those gases in the blood is altered. This can easily be demonstrated.

If the animal is breathing pure oxygen instead of air, the apnoea after adrenaline starts at about the same point, though we have

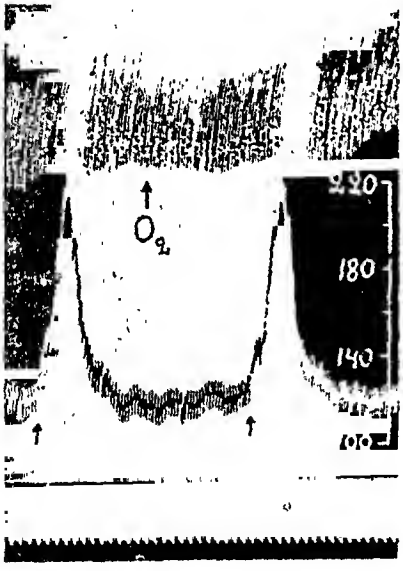
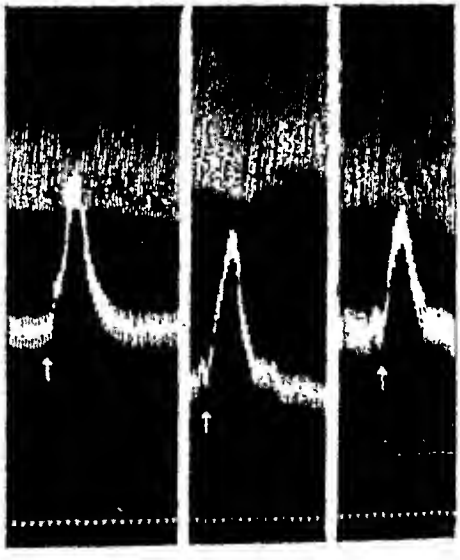


Fig. 1. Cat 2.6 kg. Upper curve respiration (body plethysmograph), lower curve blood-pressure. Time marking 10 sec. The lower arrows mark the injection of 20 μg of adrenaline intravenously. At upper arrow the cat is given to breathe pure oxygen spontaneously.



A B C

Fig. 2. The same animal as in fig. 1, the curves showing the effect of an intravenous injection of 10—20 μg of adrenaline when breathing spontaneously *A* air, *B* 5.2 per cent carbon dioxide and 22 per cent oxygen and *C* 9.1 per cent carbon dioxide and 19 per cent oxygen.

sometimes observed a short delay. From our point of view this might be explained by an earlier observation (EULER, LILJESTRAND and ZOTTERMAN 1939), according to which, changes in the oxygen tension produce very prompt changes in the electric response from the sinus nerve, whereas the effect of carbon dioxide appeared more slowly. When the blood pressure is raised by adrenaline during air breathing, both oxygen want and carbon dioxide accumulation should be counteracted; during oxygen breathing no oxygen want exists. The main influence of oxygen breathing is, however, that it greatly increases the duration of the apnoea (Fig. 1). Since oxygen want stimulates respiration exclusively from the carotid and aortic bodies, the prolongation of the apnoea with oxygen seems to indicate that the apnoea is closely connected with the activity of the chemoreceptors. On the other hand, the apnoea can be somewhat shortened though not abolished, if the animal is breathing a gas mixture poor in oxygen. In a similar way, stimulation of respiration with carbon dioxide can reduce the effect of adrenaline on respiration more or less completely (Fig. 2).

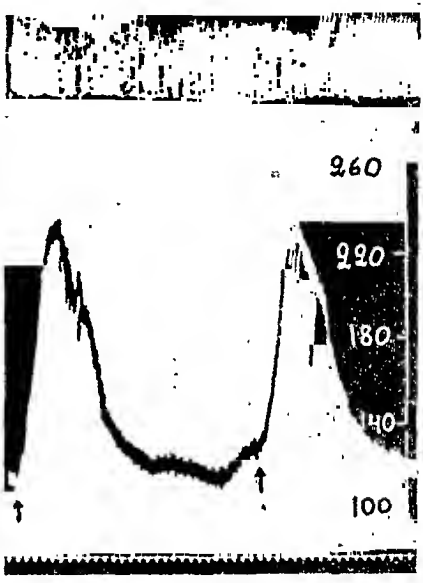


Fig. 3. Cat 3.9 kg. Arrangement as before. Both sinus nerves and both vagi cut. The animal is breathing pure oxygen spontaneously. At arrows injection of 40 μg of adrenaline intravenously.

In confirmation of HEYMANS and BOUCKAERT and WRIGHT, we too have found that only a very small immediate adrenaline effect on respiration is left, if both vagi are cut and the sinuses denervated. It seemed of interest that this small residue was still observed during oxygen inhalation (Fig. 3). It seems natural to assume that it is due to a central effect, though the detailed mechanism is unknown.

II. Action Potentials from the Chemoreceptive Fibres of Sinus Nerve.

If our interpretation is correct, it must be expected that the adrenaline apnoea is accompanied by a decrease in the action

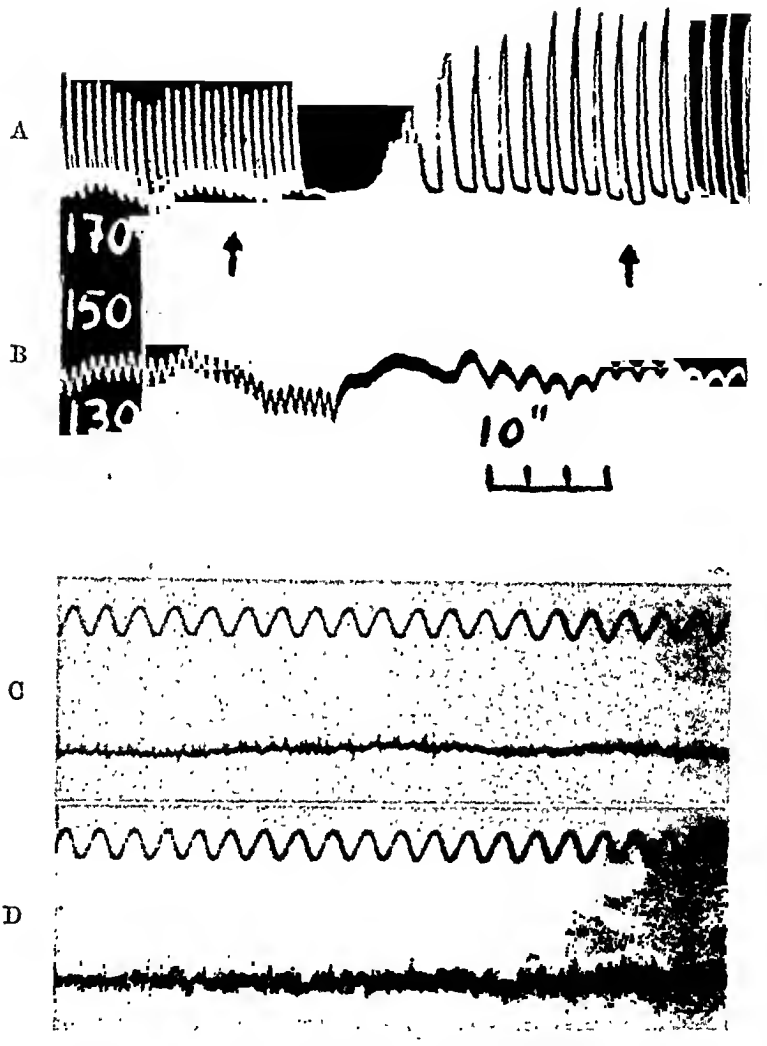


Fig. 4. Cat 3.2 kg. *A* respiration (pneumograph), *B* blood-pressure. The two lower curves show the impulse traffic in the sinus nerve (most pressure fibres eliminated) *C* during overventilation (first arrow) and *D* when spontaneous respiration is regained (second arrow). Time marking on the film strips $^1/_{10}$ sec.

potentials from the chemical fibres of the sinus nerve. In order to test this we recorded the action potentials from the sinus nerve after reducing the pressure fibres in the way described before (EULER, LILJESTRAND and ZOTTERMAN 1939). Fig. 4 illustrates that artificial overventilation with air decreased the number of potentials considerably and there can be no doubt that the majority of the impulses are elicited by chemical stimulation. From Fig. 5

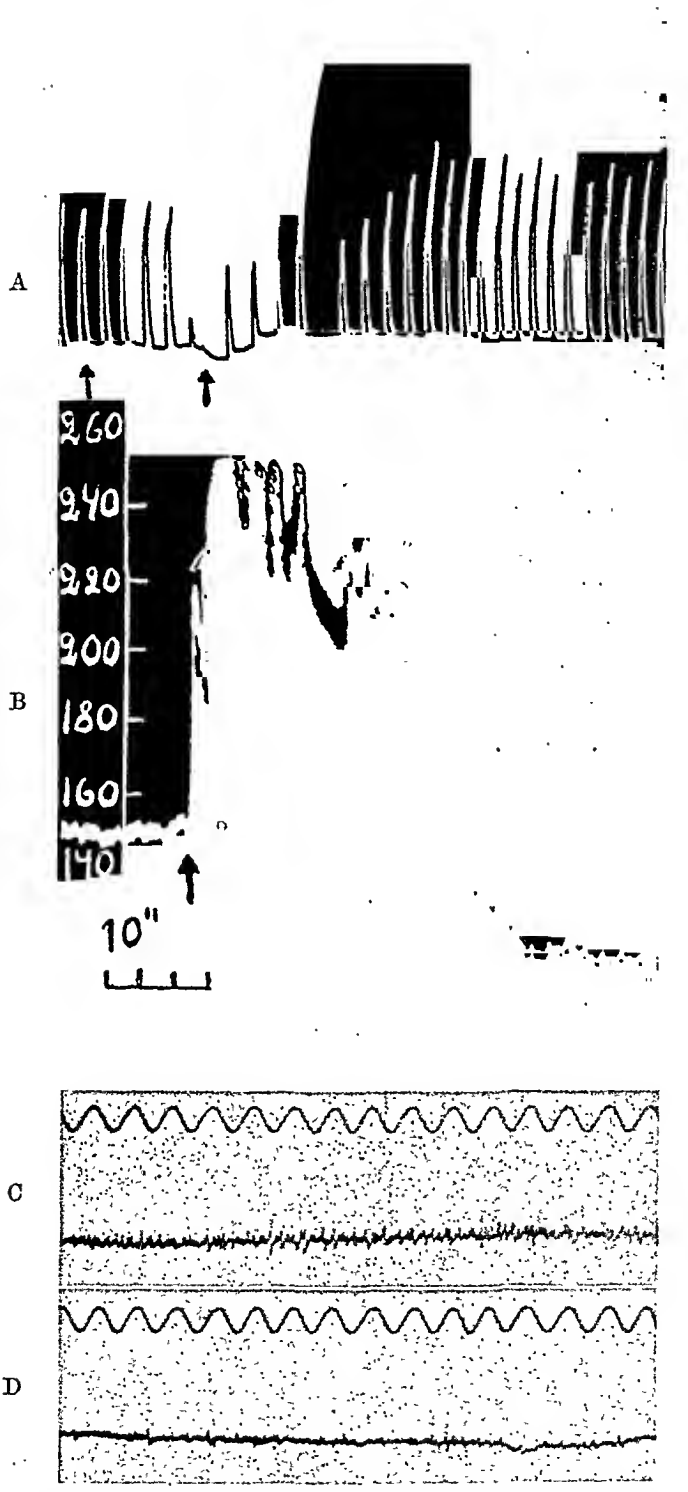


Fig. 5. The same animal as in fig. 4. The effect of intravenous injection of 40 μg of adrenaline (at lower arrow). The cat was breathing air spontaneously. *C* shows the activity in the carotid sinus nerve (most pressure fibres destroyed) before the injection (first upper arrow) and *D* immediately after the initial rise of the blood pressure (second upper arrow).

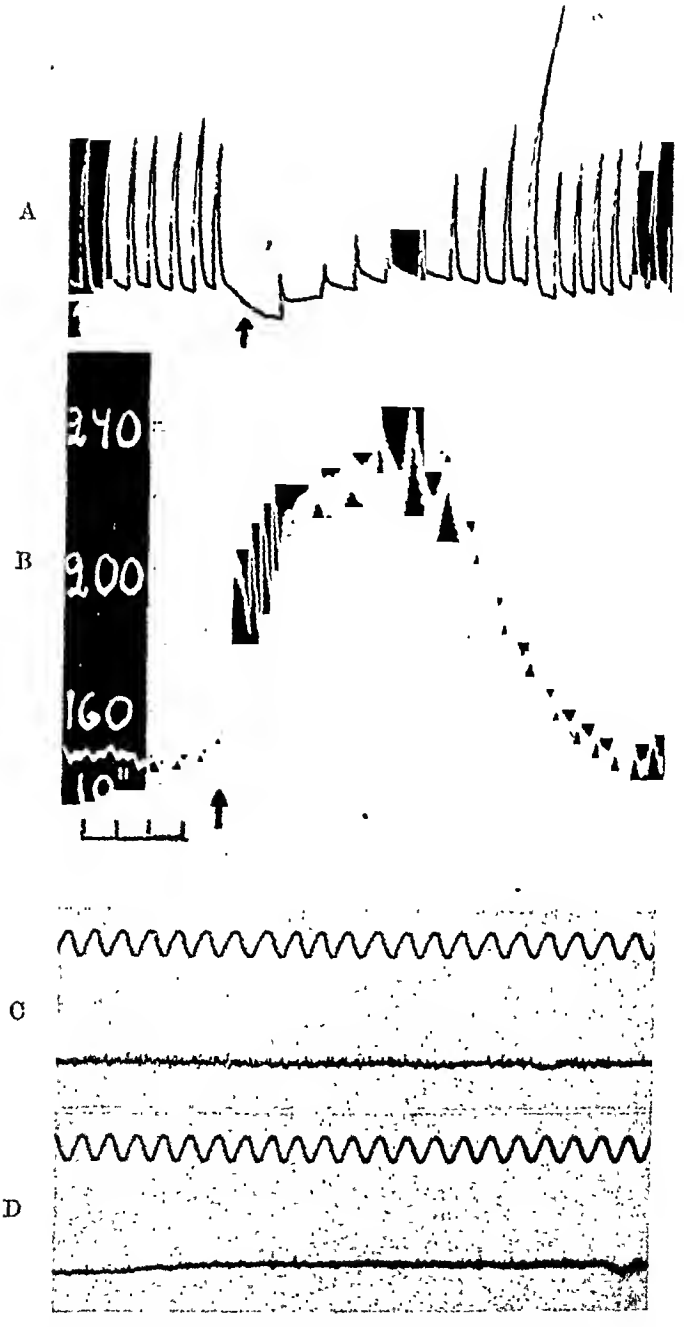


Fig. 6. The same animal as in fig. 4. The effect of 40 μg of adrenaline when the cat is breathing pure oxygen spontaneously. Note that the apnoea now is much prolonged and that the activity in the carotid sinus nerve *C* before (corresponding to first upper arrow) and *D* after the injection (at second upper arrow) is less than when the cat was breathing air.

it can be seen that the number of chemical potentials during the beginning of the adrenaline apnoea is still further reduced. After oxygen inhalation (Fig. 6) there are only very few potentials before the injection, and the activity is further depressed at the start of the apnoea. We have repeated these experiments several times and conclude, therefore, that the action potentials of the chemical fibres of the sinus nerve are greatly reduced in number during the start of the adrenaline apnoea. In our opinion this gives a definite proof that the apnoea is due to a diminution or an abolition of a reflex stimulation of respiration, and not to an inhibitory effect from the baroceptors upon the centre.

III. Can Apnoea be Produced by Section of the Sinus and Vagus Nerves?

Since adrenaline apnoea must be considered to be the result of an instantaneous elimination of the chemoreceptor activity of the sinus and aortic regions, it seemed desirable to investigate whether apnoea could be induced if the sinus and vagus nerves were cut as quickly as possible. For this purpose, the four nerves were laid free and thin threads were placed underneath them. They were lifted carefully, avoiding any stretching, and cut with scissors at a given signal. We started by cutting the two sinus nerves. As Fig. 7 demonstrates, respiration decreases immediately. This is in accordance with the results of EULER and LILJESTRAND (1936) who showed that exclusion of the sinuses regularly leads to a diminished respiration. When the vagi were cut a few minutes afterwards there followed an apnoea of short duration.

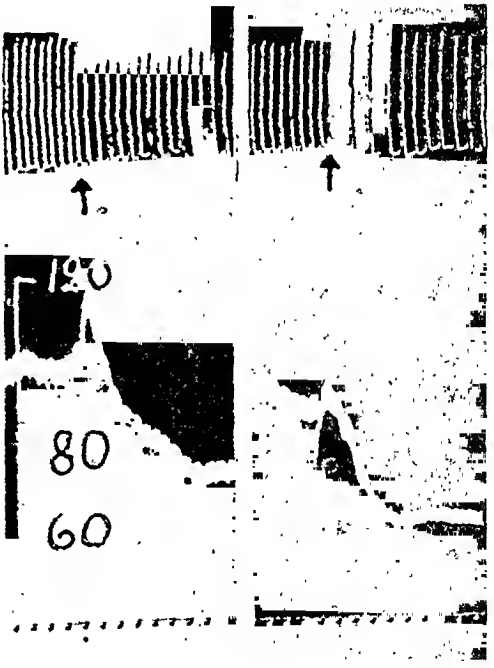


Fig. 7. Cat 2.6 kg. Upper curve respiration (pneumograph), lower curve blood-pressure. Time marking 10 sec. At the first arrow both sinus nerves cut simultaneously, at the second arrow both vagi cut simultaneously.

We then tried to cut all four nerves immediately after each other. The result (Fig. 8) was that an apnoea developed which led to the death of the animal. This is the "respiratory death" observed by WITT, KATZ and KOHN (1934), who also observed a temporary apnoea after the denervation. They suggest that the fall of the blood-pressure to low levels contributed to the depression of the respiratory centre. In good agreement with their experience is the considerable decline of the blood-pressure in our experiment. We think that this can be explained in the following way. The apnoea leads to oxygen want, and since no pressor reflexes are evoked from the sinus and aorta regions any more, the oxygen shortage will cause a depression of the vasomotor centre, and this in its turn leads to the respiratory standstill. In order to test this hypothesis, we repeated the experiment on another cat, but during oxygen breathing (Fig. 8). The result is a rapid rise of the blood-pressure, and respiration has soon resumed the usual appearance after vagotomy, but there is at first a period with decreased respiration. We think that, on the whole, these experiences lend further support to our conception of the adrenaline apnoea as the result of a temporary reduction, or an elimination, of the peripheral chemoreceptor activity.

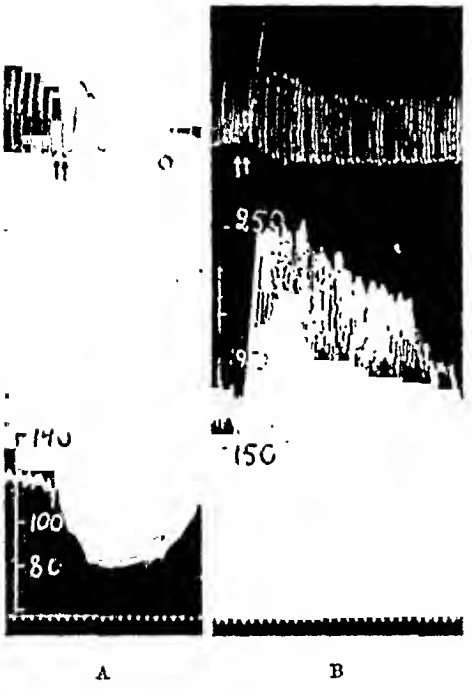


Fig. 8. Upper curve respiration (pneumograph), lower curve blood-pressure, time marking 10 sec. *A* Cat, 3.0 kg, is breathing air spontaneously. The first arrow marks the simultaneous cutting of both sinus nerves and the second arrow of both vagi. *B* Cat 3.9 kg, is breathing oxygen spontaneously. The two arrows mark the section of the nerves as in *A*.

Summary.

Adrenaline apnoea is increased by inhalation of oxygen. It can be decreased more or less, if the animal is breathing gas mixtures poor in oxygen or rich in carbon dioxide.

During the apnoea, the chemical impulses from the sinus nerve are greatly reduced in number. Respiration can be depressed or apnoea provoked if the vagi and the sinus nerves are cut within a short time of each other.

The results obtained cannot be reconciled with the hypothesis that adrenaline apnoea is due to a direct inhibition from the baroceptors of the sinus and aortic regions. In our opinion, adrenaline apnoea is the result of a reduction of the activity of the chemoreceptors.

This investigation has been aided by a grant from the Therese and Johan Andersson Memorial Foundation.

References.

BJURSTEDT, H., and U. S. v. EULER, Acta physiol. scand. 1942. *4*. 23.
BJURSTEDT, H. and C. M. HESSER, Ibidem 1942. *4*. 5.
BORUTTAU, H., Pflüg. Arch. ges. Physiol. 1899. *78*. 115.
EULER, U. S., and G. LILJESTRAND, Skand. Arch. Physiol. 1936. *74*. 101.
EULER, U. S., and G. LILJESTRAND, Ibidem 1937. *77*. 191.
EULER, U. S., G. LILJESTRAND and Y. ZOTTERMAN, Ibidem 1939. *83*. 132.
HEYMANS, C., and J. J. BOUCKAERT, J. Physiol. 1930. *69*. 254.
MELLANBY, J., and A. ST. G. HUGGETT, Ibidem 1923. *57*. 395.
OLIVER, G., and E. A. SCHÄFER, Ibidem 1895. *18*. 230.
ROBERTS, Ff., Ibidem 1921. *55*. 346.
RUDBERG, T., Skand. Arch. Physiol. 1938. *79*. 8.
RUDBERG, T., Acta physiol. scand. 1940. *1*. 89.
SAMAAN, A., and G. Stella, J. Physiol. 1935. *85*. 309.
WISING, P., Acta med. scand. 1937. *91*. 159.
WITT, D. B., L. N. KATZ and L. KOHN, Amer. J. Physiol. 1934. *107*. 213.
WRIGHT, S., J. Physiol. 1930. *69*. 493.
ZOTTERMAN, Y., Skand. Arch. Physiol. 1936. *75*. 105.

From the Department of Physiology, University of Lund.

Ultrafiltration as a Method of Preparing Blood Plasma for Quantitative Estimation of Histamine.

By

NILS G. EMMELIN.

Received 4 May 1945.

Chemical methods unfortunately are not sensitive enough for a quantitative estimation of histamine in blood plasma. Because of the low concentration of histamine it is necessary to use biological methods. Plasma, however, contains other biologically active substances, which affect the test objects, thus interfering with the assay. Prior to the assay these substances must be removed through an intensive and time-consuming process of chemical extraction. BARSOUM and GADDUM (1935) devised such a method of extraction, and with this method it was found that plasma contains histamine (BARSOUM and GADDUM 1935, ANREP and BARSOUM 1935). The Barsoum-Gaddum method was later modified by CODE (1937).

During experiments on the histamine content of aqueous humour it was found that this fluid can be tested directly on the guinea-pig ileum, without any previous chemical treatment (EMMELIN and PALM 1944). As the aqueous humour is considered by some authors to be an ultrafiltrate of blood plasma, it might be of interest to investigate whether an ultrafiltration of plasma can yield a solution, the histamine content of which can be tested directly on the isolated guinea-pig gut.

Experiments.

As a test object we used the isolated guinea-pig ileum, suspended in Tyrode solution in the usual way. Each sample was divided in two portions. One of these was extracted according to CODE

(1937), the other was ultrafiltrated. We used a method of ultrafiltration described by REHBERG (1943). The sample is placed in a cellophane tube surrounded by a basket of metal wire; the filtration pressure is obtained by centrifugation. Of the different cellophane tubes of Rehberg's, the most impermeable ones were used in our experiments. The samples were centrifuged at a speed of 3,000—3,500 rev. per min. during 30—120 minutes.

Experiments with a histamine solution of known concentration. Preliminary experiments were made with a Tyrode solution containing 100 γ histamine base per litre and 3.5—7.0 % egg albumen. Table I shows a comparison between extracted and ultrafiltrated samples. Control experiments proved that histamine was contained neither in the extract nor in the ultrafiltrate of a Tyrode solution with only egg albumen added. From the table it can be seen that about 90 % of the histamine could be recovered from the extracts, about 100 % from the ultrafiltrates.

Table I.

Tyrode solution containing 100 γ histamine per litre and 3.5—7.0 % egg albumen.

	Experiment no.	1	2	3	4	5	6	7	8	9	mean value
Histamine content γ/l	extraction	95	90	70	90	90	95	80	105	90	89
	ultrafiltration	105	105	100	100	110	105	95	105	100	103

Table II.

Guinea-pig plasma.

	Experiment no.	1	2	3	4	5	6	7	8
Histamine content γ/l	extraction	235	240	160	140	170	250	320	155
	ultrafiltration	250	260	150	150	185	240	330	165

Table III.

Rat plasma.

	Experiment no.	1	2	3	4	5	6	7
Histamine content γ/l	extraction	150	145	110	315	140	190	155
	ultrafiltration	150	150	110	330	160	215	175

Table IV.

Rabbit plasma.

Experiment no.		1	2	3	4	5	6	7	8	9	10	11	12
Histamine content γ/l	extraction	350	200	565	800	150	1200	800	800	150	1900	140	600
	ultrafiltration	310	200	555	800	150	1350	800	810	175	1900	145	620

Experiments with blood plasma. Guinea-pigs, rats and rabbits were used as experimental animals. Rabbit blood was obtained from an ear vein. Guinea-pigs and rats were anaesthetized with ether, and blood was withdrawn by heart puncture. Heparine was used as an anticoagulant. The blood was centrifuged during 20—45 minutes at 3,500 rev. per min. and plasma was collected.

Fig. 1. Isolated guinea-pig ileum, suspended in 2 ml Tyrode solution.
1) 0.020 γ histamine is added.
2) 0.14 ml extract of guinea-pig plasma.
3) 0.024 γ histamine.
4) 0.14 ml extract.
5) 0.14 ml ultrafiltrate of plasma.
6) 0.05 ml plasma. 1.0 ml of the extract corresponds to 1.0 ml plasma.

Tables II, III and IV summarize these experiments. The tables show that the results of the two methods agree *fairly* well. It seems reasonable to expect the ultrafiltrations to give somewhat higher values than the extractions. The loss of histamine is probably greater with the chemical procedures than with the ultrafiltration; the preliminary experiments with a solution of known histamine content point in this direction. Besides, the samples are not entirely comparable; the histamine concentration of the

extracts is expressed in terms of γ per litre plasma, that of the ultrafiltrates in terms of γ per litre ultrafiltrate.

Figure 1 exemplifies an estimation of the histamine content in extracted and in ultrafiltrated guinea-pig's plasma (experiment no. 5 in table II). This figure also shows that the histamine content cannot be estimated by adding the untreated plasma to the gut. From our experiments it is evident that the interfering substances can be eliminated not only by extraction but also by ultrafiltration; they are obviously to be found amongst the plasma components of large molecular size.

Summary.

Ultrafiltration of plasma from guinea-pig, rabbit or rat gives a solution, the histamine content of which can be estimated directly on isolated guinea-pig ileum. Compared with the usual chemical extraction this method is simpler and takes less time. It is also less drastic. Histamine obtained by this method must have been present in plasma in a free state or as a very labile compound.

References.

ANREP, G. V., and G. S. BARSOUM, J. Physiol. 1935. *85.* 36 P.
BARSOUM, G. S., and J. H. GADDUM, Ibidem 1935. *85.* 1.
CODE, C. F., Ibidem 1937. *89.* 257.
EMMELIN, N., and E. PALM, Acta Ophtalm. 1944. *22.* 117.
REHBERG, P. B., Acta Physiol. Scand. 1943. *5.* 305.

From the Physiological Department, Karolinska Institutet, Stockholm.

The Piperidine Output in Urine During Muscular Work.

By

U. S. v. EULER.

Received 30 April 1945.

In previous communications it has been shown that piperidine occurs as a normal constituent of human urine (EULER, 1944 a, b, 1945). The results obtained with urine from school-boys before and after the gymnastics training hour suggested that muscular work may increase the output of piperidine in urine. In the present paper some results will be presented which deal with the excretion of piperidine in human urine during controlled muscular work.

Methods.

The quantitative determination of piperidine was made according to the methods described in a preceding paper (EULER, 1945). As a rule the determinations were made colorimetrically with the naphthoquinone reagent, but in several instances the results were checked by biological determination on the cat's blood pressure or on the isolated intestine of the rabbit. The agreement between the results obtained by the different methods was constantly good.

The experimental subjects were told to empty their bladder as completely as possible before the beginning of the work and to note the exact time. After the termination of the work the subjects had to micturate again and the time interval between the two micturations was recorded. Since the amount of urine obtained from the short periods of work was too small for collection and extraction, the urine was taken from longer periods, usually 1—3 hours. From the total piperidine amount the estimated output during rest was subtracted. This was calculated on the assumption that the output per minute was 0.01 mg piperidine at rest, a figure which may be regarded as safe and is slightly in excess of the rates actually determined. The output during work is then expressed in the amount per hour for comparison with the resting value. The error introduced in the results by this method of calculation will probably not be of any appreciable importance.

Most of the samples were obtained from athletes running at various

speeds[1] or working at a fixed rate on the bicycle ergometer. The degree of muscular work was also controlled by determination of the oxygen consumption in a number of cases.

In other experiments samples were taken from single subjects or groups of subjects performing more or less heavy work in gymnastics or athletic training.

Control determination of the piperidine output during periods of rest were made on urine collected from the night's sleep or from shorter periods of day-time rest.

Of the samples 40 ml were subjected to 3 hours' continuous fluid extraction with ether according to the method previously described and the ether solution extracted with acidulated water. This extract could be used for direct colorimetrical determination. When biological determinations were made, larger samples of urine had to be used for extraction. The figures of piperidine excretion are given in terms of piperidine hydrochloride. They are not corrected for the small deficit due to incomplete extraction.

Results.

I. Piperidine excretion in urine during muscular work in single subjects with control of work or oxygen consumption.

In the following table I the results from 3 single subjects — all of them well-trained athletes — are summarized and the piperidine output during rest and work shown.

Table I.

Name	Date 1944 —45	Running work/speed km per hour	Oxygen consumption l/min.	Period of work min.	Urine sample interval min.	Urine volume ml	Piperidine mg total	Piperidine output mg/hour	
								rest (sleep)	work
H...	19/12	9—15	2.7—3.7	33	120	83	2.6		(3.1)[1]
H...	8/1	10—15	2.7—3.6	20	90	100	1.1		(1.1)[1]
H...	19/1				540	270	2.4	0.27	
H...	8/1				555	265	1.8	0.19	
H...	13/1				480	235	4.1	0.51	
H...	18/1				525	265	1.6	0.18	
H...	21/1				360	250	1.9	0.31	
H...	25/1				525	265	2.4	0.27	
H...	13/1	17	4.2	15	90	70	2.1		5.4
H...	6/2	17	4.2	9	60	60	1.0		3.3
H...	7/2	18	4.6	9	70	37	1.3		4.5
H...	9/2	18	4.5	9	55	100	1.5		6.7
H...	14/2	17	4.5	12	70	37	2.4		9.4
H...	11/3	20		5	55	52	0.7		2.8
H...	7/3	19	4.7	8	40	100	1.1		5.6
H...	9/3	20	4.7	6	80	50	1.4		6.0
[1] Not included in the average						Average (H)		0.29	5.5

[1] My thanks are due to Prof. E. Hohwü-Christensen for his kindness in placing his material at my disposal and to those who gave urine samples.

Name	Date 1944 —45	Ergometer work	Oxygen consumption l/min.	Period of work min.	Urine sample interval min.	Urine volume ml	Piperidine mg total	Piperidine output mg/hour	
								rest (sleep)	work
J....	20/3				540	329		0.50	
J....	22/3				540	265		0.42	
J....	21/3	1440 kgm/min.		33	45	50	1.0		1.6
J....	26/3	»	2.7	30	45	39	0.7		1.2
J....	20/4	1260 kgm/min.		30	130	108	4.1		6.2
J....	23/4	»		30	130	228	2.3		2.6
J....	24/4	»		30	100	185	1.9		2.4
J....	25/4	»		30	100	67	1.2		1.0
J....	26/4	»		30	130	95	3.6		5.2
						Average (J)		0.46	2.9
M...	30/4				510	517	4.1	0.48	
M...	3/5				510	474	4.7	0.56	
M...	20/4	1620 kgm/min.		10	150	100	1.8		2.4
M...	23/4	»		10	210	107	3.9		11.4
M...	24/4	»		10	150	101	2.0		3.6
M...	25/4	»		10	210	185	3.3		7.8
						Average (M)		0.52	6.3

From the Table I it is evident that the piperidine output in urine is considerably increased as a result of moderate or heavy muscular work as compared with the excretion during rest. Thus the output in the subjects H and M amounted to 5.5—6.3 mg per hour (hard work) and in subject J 2.9 mg per hour (moderate work) as against a resting value of 0.3—0.5 mg per hour.

II. Piperidine excretion in miscellaneous experimental subjects at work and at rest.

Determinations have been made of the piperidine output in urine in a number of persons which have been performing various kinds of work. The results are summarized in the Table II. Control determinations have been made from comparable periods of day-time rest.

The samples from the 7 juniors were collected after a road run competition of 3.5 km. The figure in the last column is the average from all seven sprinters, whose samples were mixed and treated together. The 6 juniors were healthy male medical students at ordinary room rest.

Table II.

Name	Date	Work	Period of work min.	Urine sample interval	Urine volume ml	Piperidine output mg per hour	
						rest	work
G.....	5/2			6^{30}—8^{30}	200	0.53	
G.....	6/4			6—8	78	0.51	
G.....	23/4			15^{15}—16^{15}	74	0.83	
G.....	13/2	Hard	17—18^{30}	17—18^{30}	95		4.4
G.....	21/3	Moderate	17^{30}—18^{30}	17^{30}—18^{30}	60		2.6
G.....	16/4	Light	10^{15}—11	10^{15}—11	70		1.7
7 Juniors....	22/4	Hard	10	25	580		4.6
6 Juniors....				15—17	899	0.53	

Discussion.

Though the results have shown a consistent increase in the amount of piperidine excreted through the urine in connection with muscular work, considerable variations in the single figures occur. This is no doubt partly dependent on the difficulties in emptying the bladder completely before and after the experimental period. In the case of low urine volumes the errors introduced will be considerable and this is probably the explanation why the three lowest urine volumes corresponded to the lowest piperidine figures in the experimental subject J. The lowest figures for this reason are somewhat doubtful.

The highest figures, single and average, are found in the series where the intensity of the work done was high. Since the high values were also obtained when the period of rest preceded the work and the bladder emptied shortly after the end of the work, it is inferred that the excretion sets in very rapidly. This is in agreement with the results of experiments where piperidine was taken by mouth and its rate of excretion determined. Already after 15 minutes a definite increase in the piperidine output could be demonstrated.

Summary.

The piperidine output in urine at rest was found to be from 0.3—0.5 mg per hour (sleep) to 0.5—0.8 mg per hour at daytime rest in healthy young males.

In connection with moderate or hard work the piperidine excretion in urine was greatly augmented. Average figures exceeding 6 mg per hour were obtained.

This work has been aided by a grant from the Therese and Johan Andersson Memorial Foundation.

References.

EULER, U. S. v., Nature 1944. *154*. 17.
EULER, U. S. v., Acta Physiol. Scand. 1944. *8*. 380.
EULER, U. S. v., Acta Pharmacol. 1945. *1*. 29.

ACTA PHYSIOLOGICA SCANDINAVICA
VOL. 9. SUPPLEMENTUM XXVII.

FROM THE LABORATORY FOR THE THEORY OF GYMNASTICS, UNIVERSITY OF COPENHAGEN

CENTRAL AND INDIRECT VISION

OF THE LIGHT-ADAPTED EYE

By

VIGGO CLEMMESEN

COPENHAGEN 1944

J. A. HANSENS BOGTRYKKERI

Denne Afhandling er af det lægevidenskabelige Fakultet antaget til offentlig at forsvares for den medicinske Doktorgrad.

København, den 27. September 1944.

H. Helweg,
h.a. dec.

PREFACE

The investigations here presented were carried out in The Laboratory for the Theory of Gymnastics, University of Copenhagen, in 1938—1943. I wish to express my most hearty thanks to the chief of this laboratory, Professor *Em. Hansen,* D. Sc. for the excellent working facilities afforded me and the great interest he has taken in this work.

Next I wish to express my sincere thanks to *Fritz Buchthal,* M. D. at the same laboratory for his living, friendly interest, his never failing willingness to support my attempts to clear up the problems of indirect vision and for his valuable criticism of this manuscript.

I am also greatly indebted to *Curt Berger,* Ph. D. (now Ithaca, N. Y.) for the original impulse to the present investigation, for placing some of his experimental arrangements at my disposal and for several stimulating discussions on visual acuity.

To Professor *E. Rubin,* Ph. D. I wish to acknowledge my gratitude for his readiness to read through and criticize the present book.

I further wish to thank Dr. *Knud Rasmussen* and *Niels Bolwig,* M. S. for their great interest in my researches.

To all my experimental subjects, especially Mrs. *R. A. Wissing* I am obliged for their painstaking assistance and to Mr. *C. Persson,* chief mechanician of the laboratory for his skillfull construction of the most of my test object apparatus.

Financial aid for the investigations was granted me by the Miss *P. A. Brandt* Fund for which I wish to acknowledge my obligation.

THE LABORATORY FOR THE THEORY OF GYMNASTICS,
UNIVERSITY OF COPENHAGEN.

October 1944. *Viggo Clemmesen.*

TABLE OF CONTENTS

Page

Introduction 1

PART I

The Physiology of Central and Indirect Vision, Previous Investigations.

Chapter I. Direct or Central Vision. Division of the Sense of Vision in Partial Functions 4

A. Light Sense 6

1. Differential Threshold 7

a) Dependence on light intensity and adaptation 7

b) Dependence on the stimulated retinal area 8

2. Intensity Threshold 11

B. Colour Sense 13

C. The Physiological Resolving Power 14

1. The Conditions for the Exact Localization of the Image Point in the Monocular Field of Vision 14

a) The influence of the optical system 14

b) Remarks on the structure of the fovea 16

The size of the receptors and their number per unit of area 16

The centripetally conducting neurones of the fovea 17

2. The minimum separabile 19

a) The dependence of the minimum separabile on the size of the test object, its relationship to the retinal mosaic and its utility as a measure for the visual acuity 20

b) The dependence of the minimum separabile on the illumination 25

Objects illuminated from before 26

Luminous objects on unilluminated ground .. 28

3. Perception of Movement 36

4. Form Sense 40

Page

D. Complex Functions 42
1. Aligning Power 42
2. Discrimination of Thicknesss of Line 44
3. Visual Acuity as Measured by Recognition of Letters 45

Chapter II. Indirect Vision 51

A. The Principles of Indirect Vision 51
1. The Optical Imagery by Indirect Vision 51
2. Remarks on the Structure of the retina outside the fovea 53
3. The Importance of the Psychological Factors, Attention and Practice, for Indirect Vision 54

B. Previous Investigations on Indirect Vision 55
1. The Angular Extent of Central Vision Determined by means of the Size of the Retinal Fixation Area 56
2. Previous Comparative Investigations on Central and Indirect Vision 59
a) The light sense by central and indirect vision.. 59
b) The colour sense by central and indirect vision 61
c) The physiological resolving power by central and indirect vision 61
The minimum separabile 61
Perception of movement and form sense 64
d) Variation of the complex functions by central and indirect vision 64
Aligning power 64
Discrimination of thickness of line 66
The minimum legibile 66

C. Theoretical Explanation of the Difference between the Characteristic Features of Central and Indirect Vision 67
Electro-physiological experiments on central and indirect vision 72

PART II

Author's Investigations. Comparative, Sense-physiological Experiments on Central and Indirect Vision.

Survey of Part II 78

Chapter III. Experimental Methods 85

A. General Experimental Conditions 85
Subjects 85
Light adaptation 86
Artificial pupil 86
Fixation of the head of the subject 86

Page

Experimental distance 86
Fixation target 87
The illumination of the test object 88
Calculation of the illumination of the stimulated retinal area 90

B. Special Arrangements for Examining Individual Visual Functions 91
1. Arrangements for Examining the Physiological Resolving Power 91
a) The minimum separabile 91
Luminous squares on unilluminated ground .. 91
Unilluminated squares on luminous ground ... 96
b) Perception of movement 96
c) Form sense 98
2. Arrangements for Experiments on Complex Functions 99
a) Aligning power 99
b) Discrimination of thickness of line 99

C. Course of Experiments and Calculation of Results .. 101
Definitions of the thresholds read 102

Chapter IV. Experiments on the Physiological Resolving Power 1. *Experiments on the minimum separabile* 105

A. The minimum separabile for Luminous Points on Unilluminated Ground at Different Retinal Eccentricities 105
The accuracy of the experiments 110

B. The Dependence of the minimum separabile on the Retinal Meridian 115

C. The Influence of Attention and Fixation Target on the minimum separabile 119

D. The Dependence of the minimum separabile on Illumination 123
1. The Influence of Intensity 123
2. Continuous Illumination and Momentary Illumination 127

E. The Dependence of the minimum separabile on the Size of the Squares 132
1. Luminous Squares on Unilluminated Ground 133
a) Central vision 133
b) Indirect vision 138
2. Unilluminated Squares on Luminous Ground 141
a) Central vision 141
b) Indirect vision 143

Page

3. The minimum separabile for Twin-objects Illuminated from before 144

Chapter V. Experiments on the Physiological Resolving Power 2. *Experiments on the Perception of Movement* 148

Chapter VI. Experiments on the Physiological Resolving Power 3. *Experiments on Form Sense* 151

A. Form Sense at Various Retinal Eccentricities 152
The accuracy of the experiments 153

B. The Dependence of the Form Sense on the Retinal Illumination 156

Chapter VII. Experiments on Complex Functions 1. *Experiments on the Aligning Power* 159

A. The Aligning Power at Various Retinal Eccentricities 160
The accuracy of the experiments 161

B. The Dependence of the Displacement Threshold on the Length of the Slit 163

Chapter VIII. Experiments on Complex Functions 2. *Experiments on Discrimination of Thickness of Line* 166

A. Discrimination of Thickness of Line at Various Retinal Eccentricities 167
The accuracy of the experiments 170

B. The Influence of Retinal Illumination on the Discrimination of Thickness of Line 173

C. The Influence of the Dimensions of the Object on the Discrimination of Thickness of Line 173
1. The Influence of the Length of the Line 173
2. The Importance of the Width of the Line 175

Chapter IX. Appendix. *Experiments on Troxler's Phenomenon* 177

A. Troxler's Phenomenon and the Theories Advanced in Explanation of it 177

B. Experiments on the Influence of the Retinal Illumination 180

Conclusive Remarks on Central and Indirect Vision 183
Central vision 183
Indirect vision 185

Summary 188

Danish Summary, Résumé 193

References 197

INTRODUCTION

When the gaze is directed towards a point and the fixation of the point is continued, any object situated in the field of vision will be seen less distinctly than at the fixation point. *Hirschberg* (1878) compared the visual field to a painting on which only a very small central area (subtending an angle of 1^0) is carefully painted, the remainder barely sketched, and that more and more roughly the nearer one gets to the edge of the picture. Moreover, only in a zone within $8—10^0$ from the centre of the picture saturated beautiful colours are seen, while further from the middle the colours disappear one after the other in regular succession, until the extreme periphery shows quite colourless — grey painted on grey.

When one wishes to scan more closely the single objects in the visual field, these must be fixated successively — a process which normally functions unconsciously.

The part of the retina on which the image of the fixation point falls is known as the seat of the direct or central vision (*Purkinje* 1825[4], *Helmholtz* 1896[36]) while the indirect vision is confined to the remaining regions of the retina. Only the terms direct or central and indirect vision — as defined above — are used. The term — peripheral vision — is not desirable, as this naturally refers to the periphery, but does not characterize the less eccentric vision especially the paracentral.

How large an area of the retina is occupied by direct vision is still undecided. Some authors maintain that vision is uniform throughout the fovea, while others have found that direct vision is confined to a much smaller area.

Further investigations on the boundaries of direct vision on the retina are thus desirable.

It can be regarded as quite definite that by indirect vision the visual acuity gradually diminishes with increasing eccentricity in the field, but is somewhat different in the two retinal meridians, as the visual acuity decreases more slowly in the horizontal than in the vertical meridian. How rapidly the indirect vision is decreased is not definite. It is not known whether the difference found is due to the method employed or to individual differences in subjects tested.

The theories which in the course of time have been propounded to explain the difference between indirect and central vision, have naturally been based upon conditions — optical, structural or otherwise — which are known to differ, corresponding respectively to the fovea and the remaining retina. Which condition carries most weight differs with the different authors. Some regard the distinctness of the retinal image as the most essential, others — and perhaps the majority — think that structural conditions, partly the relative distribution of the cones, partly the »coupling« of the cones to larger units by horizontal connections can explain all features of indirect vision. Finally there are authors who consider psychological factors such as attention and training as the most important. While other explanatory possibilities can hardly be imagined, information on an experimental basis concerning the relative importance of the individual factor is lacking, especially whether indirect vision can be explained in the same way in all zones of the retina.

It has therefore been thought desirable to perform new experimental investigations on central and indirect vision, the results of which are shown in this book.

In Part I an account of the physiology of central and indirect vision is given. Part II states the results of certain experiments which attempt to throw light on the difference between central and indirect vision, hereby going deeper into the problems surrounding indirect vision and — perhaps — solving some of them.

PART I

THE PHYSIOLOGY OF CENTRAL AND INDIRECT VISION, PREVIOUS INVESTIGATIONS

Chapter I

DIRECT OR CENTRAL VISION, DIVISION OF THE SENSE OF VISION INTO PARTIAL FUNCTIONS

If indirect vision is to be compared with direct vision to account for the causes of the special features of the former, it is natural to measure with a suitable object how the acuteness of vision differs in various zones of the retina. In these experiments only objects with a very simple retinal image are to be used so that they can be set in relation to the structure of the retina. Further they must allow the variation of only one experimental condition at a time. The experiments are to be performed with one eye only to prevent any co-operation between the two eyes.

No attempt is made here or in the following presentation to explain the sense phenomena by the centrally-located processes which are always present, because in sense physiology one tries to localize phenomena to the peripheral sensory-apparatus (*receptors* and *conducting neurones*) before the possibility of central localization can be discussed. Thus, the psychological component of the visual act is left out of consideration as long as possible.

To discover which methods are best suited to comparative experimental investigations on direct and indirect vision it will be necessary to perform an analysis of the physiological component of the sense of vision. This can be divided according to different principles, depending on the definitions established for the individual components and their relative limitations. While the majority of authors, from *Aubert* (1865[7]) to *Duke-Elder* (1932[**4]) and *Adler* (1934) are agreed upon a rough classification of the sense of vision into light sense, colour sense, and form sense, they remember a

further intensive subdivision, and are content with explaining the physiological conditions for the correct recognition of certain simple experimental figures, by which definite sides or »partial functions« of the sense of vision can be investigated. The following partial functions which hitherto have been investigated are intensity threshold (min. perceptibile, Reizschwelle), min. visible, differential threshold (min. distingvibile), min. separabile (for points, squares, and lines), resolving power. visual acuity, visual acuity as measured by recognition of letters (min. legibile), form sense (min. cognoscibile), aligning power (space threshold) and movement threshold. The functions mentioned can quite certainly be classified according to definition, but it is not impossible that many of them rest on same physiological basis, while the condition of others appears to be so complex and involved that they can only with difficulty be used for experimental investigations.

In the following an attempt is made to group the partial functions according to the individual prerequisites so as to form a starting-point for comparison between central and indirect vision.

The physiological component of the visual sense is divided into three main functions.

A. Light sense, defined as the ability to recognize the differences in intensity, measured by the discrimination factor.

B. Color sense. The ability to recognize the colour (wave length) of the light, measured by sensitivity to change in wave length.

C. The physiological resolving power, the ability to recognize the exact position in the monocular field of vision of the image-point, measured by the minimum separabile for two points.

The physiological resolving power can also be measured by the movement threshold or by the form sense. Min. separabile is chosen as measure due partly to its suitability, partly to convention.

A. Light Sense.

Light sense is defined as the ability to recognize the differences in light intensity, an ability which will only be dependent on the sensitivity of the receptors, and will be independent of a possible subdivision of the sense organ into different receptors — unless these are able to cooperate. Neither will the optical imagery have any influence on the light sense. Even a single sense cell, with its corresponding afferent neurones may record changes in the illumination. In conformity with this a rise in the frequency of discharge from the nerve fibre of a single receptor cell has been observed on the illumination being increased. (*Hartline, Keffer & Graham* 1932).

The intensity discrimination, as mentioned in the definition, is used as measurement of the light sense. It is expressed by the discrimination factor (the ratio $I/\Delta I$ i. e. the start intensity divided by the intensity discrimination threshold (differential threshold or light difference)). A sense organ, consisting of only one sense cell with its corresponding neural connections cannot register more than one intensity at the same time. Light sense must therefore in this case be measured by the »successive differential threshold« i. e. by sensitivity to successive changes in intensity. As a special instance of intermittent stimulus the determination of *fusion frequency of flicker* may be mentioned. By this the rate of presentation of the successive stimuli which is necessary to produce complete fusion is found.

Only when the visual organ consists of at least two independent anatomically and functionally separated units (each perhaps consisting of several receptors) will there be a possibility of simultaneous comparison of two light intensities, and thereby of determining »the simultaneous differential threshold«. Even two receptor units, of which one is more illuminated than the other, will be able to give a certain one-dimensional spatial orientation of the direction of the source of light.

In the case where the start intensity is 0, the differential threshold will be identical with the intensity threshold for

light, (minimum perceptibile or light minimum). Viewed superficially the distinction between the differential and intensity threshold is only of slight importance. On the other hand, it will be seen later that this distinction is of a great practical importance (see p. 23).

It is often proved that the differential threshold is dependent on the light intensity. Furthermore, the size of differential as well as intensity threshold has proved itself a function of the stimulated retinal area. In the following a reference — in outline — of the results of the investigations found in literature on the subject will be attempted.

1. Differential Threshold.

a. Dependence on light intensity and adaptation.

According to the *Weber-Fechners Law* intensity discrimination is constant, independent of the light intensity. After experiments, among which those of *Aubert* (1865) and *König & Brodhun* (1888), it is generally regarded as definite that this law holds good for medium intensities for a light-adapted eye not blinded by glare. (*Hecht* 1925, 1935). For lesser as well as higher intensity the discrimination factor is less. (The differential threshold greater). The optimal size of the differential threshold amounts to about ½-2 per cent of the intensity and can only be obtained with large test areas, so that the differential threshold found must be regarded as a function of the intensity discrimination for a mixed central and indirect vision, (further details see p. 10). (Although this section is mainly concerned with central vision, the intensity discrimination by indirect vision will be discussed where desirable).

The majority of investigations on the differential threshold (e. g. *Fechner* (1859), *Aubert* (1865), *König & Brodhun* (1888), *Hecht* (1925, 1935)) were not performed with constant light-adapted eyes — the luminous intensity of the test object only determining the adaptation level. The only experiment in which the influence of the adaptation factor could be separated from that of the intensity on the differential threshold was

performed by *Craik* (1938). By this experiment the successive differential threshold for a circular test area (angular diameter 35°) surrounded by an adapting screen (angular diameter 45°). was determined. The differential threshold proved to be not less than 1 per cent of the start intensity if test and adaptation intensity were equal. This holds good for all intensities, especially for the light-adapted eye. If the brightness of the adaptation field is greater than that of the test object the differential threshold increases sharply up to 100 per cent of the intensity. If the opposite is the case the differential threshold increases too, but not to the same extent.

When the eye is kept constantly adapted to light the differential threshold first decreases with increasing light intensity until adaptation intensity is reached. With higher intensities the differential threshold again increases quite slowly, having remained approximately constant round its minimum (*Craik* 1938).

b. Dependence on the stimulated retinal area.

The stimulated area of the retina may influence the intensity discrimination, partly by its size and partly by its position on the retina in relation to central and indirect vision. The following pages are mainly concerned with the dependence of the size (of the area) while a discussion of the regional influence is left to chapter II (p. 59).

When the dependence of the differential threshold on light intensity is to be investigated, the light difference can either be kept constant and the size of the object varied until the threshold has just been passed, or vice versa. In the first case — the visual angle being varied — the fact that the differential threshold has been exceeded will be manifested by the object changing from invisible to visible against its background. This holds good especially for objects illuminated from before, e. g. white points on a black ground or vice versa, as the black ground or the black points will always reflect a certain percentage (7 per cent) of the light falling on them (*Hering* 1925[14]). Visibility of the objects in such a case will be con-

ditioned by the difference in brightness between black and white, that is by the intensity discrimination of the retinal area on which the image of the object falls.

Since *Tobias Mayer* (1754) it has been known that for an object illuminated from before the visual angle and the brightness necessary for the object to be visible against its background vary inversely. In the course of time a good many experiments have been performed to formulate this relation. As one of the best known the so-called *Ricco's* law (1877) shall be mentioned. According to this the product of the area and the brightness is constant for objects with a retinal image less than the fovea. The validity of *Ricco*'s law in practice will not be discussed here, we shall merely mention that originally the law was formulated as a law on the areal dependence of the intensity threshold based on experiments with white points on black ground illuminated by borrowed light, but with this assumption that the black ground reflects no light — which, however, is not the case.

Among the investigations of the differential threshold performed with constant test area and variable intensity difference those in which the eyes of the experimental subjects were not sufficiently light-adapted must be excluded (e. g. *Asher* (1897), *Guillery* (1897 a), *Lazareff* (1911), *Stiles & Crawford* (1934), *Steinhardt* (1936)). Thus only three papers remain (*Heinz & Lippay* (1928), *Craik* (1938), and *Podestà & Aeffner* (1940)), on which this representation of the dependence of the differential threshold on the area can be supported. The results obtained by the various authors are recorded in Table I, from which it appears that both the successive and the simultaneous differential threshold for an object compared with its surroundings, expressed in per cent of start intensity and intensity of surrounding field respectively, increases rapidly with decreasing size of object within the present range of measurement. The size which the stimulated retinal area must have for the differential threshold to reach its optimal value of 1/2 to 2 per cent cannot be seen from the table. It is, however, clear that the image which covers only a retinal

area corresponding to the fovea (angular diameter 90 minutes of arc*) is not large enough. It follows therefore that the optimal differential threshold cannot be attained by central vision alone, but only

Table I.

The values of the successive and simultaneous differential thresholds as a function of the size of the test object expressed in per cent of start and background intensity respectively. Light-adapted eye.

Angular Diameter of Test Object:	Successive Differential Threshold:		Simultaneous Differential Threshold:
	Heinz & Lippay (1928)	*Craik* (1938)	*Podestà & Aeffner* (1940)
7'40"			200
11'30"			113
11'37"	22		
15'20"			55
20'	15		
23'			29
35'	10		
38'			20
1°01'	5.1		
1°16'	4.1		
1°17'			5.6
1°30'		3.8	
3°03'	3.2		
36°		1	

when at least part of the indirect vision is stimulated simultaneously.

When comparing the values of the table for the simultanous differential threshold with those corresponding to the

*) To simplify comparison between distance on the retina and distance in the field of vision, all measurements in length in the retina or visual field in this book, are, as far as possible measured by visual angle, 1' being taken to be equal to 4.95 μ (according to the schematic eye of *Gullstrand*).

successive differential threshold, the latter appear to be less than the former especially with small objects. As the material available is very small and accumulated from experimental results obtained by different methods no reliable conclusion can be drawn. It is probable, however, that the simultaneous differential threshold will always be larger than the successive as the brightness of sensation of a light source (*Broca & Sulzer* (1902), *Lehmann* (1905)) and the fusion frequency of flicker (*Granit* 1936 [66]) is higher with momentary illumination than with continuous illumination.

2. Intensity Threshold.

For the receptor mosaic of the retina to record the image of an object, the image must be illuminated so much more (or less) than its surroundings that the difference in intensity is recognizable. The retinal image of a luminous point forms a light spot (diffusion circle) the intensity of which decreases steadily in all directions without any sharp boundaries (for further details see p. 15). The size of the light spot will depend not only on the size and intensity of the point but also on the illumination of the surrounding retina. A luminous point will, therefore, only on an absolutely unilluminated ground have a size independent of the ground illumination. As the intensity of the image decreases from the centre outwards, the absolute minimum in visual angle can be achieved by fixing the intensity of the point just over the threshold and at the same time avoiding illumination of the background.

We shall here assume that the intensity threshold, i. e. the sensitivity of the individual receptors is constant. This, as will be shown on pages 34, 131, 156 and 181 is only approximately correct.

Similar considerations hold good with regard to the perception of a dark point on luminous ground. If the illumination is just strong enough to cause all the receptors to be stimulated, the receptor or receptors on which the image of the dark spot falls will be stimulated under their threshold

value, and the point will thus be visible. Its visibility will be dependent on the intensity threshold only. When the background is strongly illuminated the dark image will, because of the dispersion of light in the refractive media of the eye (see p. 32), receive an illumination just above the threshold value. For this reason the size of a dark point which can just be recognized will not be a function of the intensity threshold but of the differential threshold for the intensity concerned of the less illuminated receptor (or receptors) in proportion to the background.

Experimental determinations of the size of the intensity threshold have very seldom been carried through and in any case always with dark-adapted eye, while corresponding experiments in light adaptation have apparently not been made. It is obviously impossible to draw conclusions regarding the light-adapted eye from results obtained in dark adaptation. It is, however, certain that the intensity threshold in case of light adaptation must lie on a much higher level of intensity than in case of dark adaptation.

It has often been shown, that for the dark-adapted eye, the intensity as well as the differential threshold, decreases with increasing size of object (e. g. *Graham & Goldmann* 1932, *Graham, Brown & Mote* 1939). The majority of these experiments have been performed with relatively large objects (e. g. *Henius* 1909, *Borchardt* 1914, *Pickard* 1936, *Wald* 1938). That so few (*Bouma* 1939, *Kühl* 1940), have used quite small objects is in all probability due to the lack of certainty which characterizes such experiments. This uncertainty is due, partly to the deficient fixation of the dark-adapted eye, partly to the presence of *Troxler*'s phenomenon (see p. 177) and the strongly wavering intrinsic light of the dark-adapted eye; each of these factors renders a uniform determination of the threshold difficult.

If the intensity threshold for a green point source which emits continuous light is calculated in units of energy i. e. in energy quanta per sec. the values obtained will according to *Houston & Shearer* (1930) be 9840 q. per sec. while the intensity threshold for intermittent light is 1400 to 2600 q. per sec. If the luminous point is not green (wave length 530 μμ) but red or violet a much greater quantity of light is necessary. For a relatively large object (exact size not stated) *Russel* (1917) found an intensity threshold of 200 q. per sec. for achromatic light.

According to these results the statement made by *N. Bohr* (1933),

that the absorption by a receptor cell of very few quanta — or even one — should be able to excite the sensation of vision, does not seem to be in conformity with facts.*)

B. Colour Sense.

By the colour sense of the eye we understand its ability to recognize and differentiate between colours.

It would be appropriate here to give a collected presentation of the physiology of the colour sense with a discussion of the theories of colour vision, but the limitations of the present book make this impossible. Instead a review will be given of some of the electro-physiological experimental results of recent years and of the theoretical considerations arising from them.

The first condition of a perception of colour is that the peripheral sense organ must be able to record the differences in the wave length of the light. Electro-physiological research of recent years has shown, that the nerve impulses from one single nerve cell appear to vary only in frequency, while the size of the impulses is constant (*Hartline & Graham* 1934) (*Granit* 1941 b) (see p. 72). Since the frequency is conditioned by intensity of stimulus (light intensity) and the instantaneous sensitivity of the ganglion cells to this (irritability) and by nothing else, one cannot imagine that the nerve impulses from a single cell can express the variations in the wave length of the light except by a greater sensitivity to certain wave lengths rather than others. It is theoretically possible that the type of discharge is a function of the wave length of the light, but this has never been proved (*Granit* 1941 c). The only possible explanation of a compounded trichromatic colour perception appears to be to ascribe to the receptors a selective colour sensitivity, so that colour perception results from a synthesis of impulses from different ganglion cells for each point in the field of vision.

The eyes of the lower animals (e. g. Limulus, (*Hartline* 1934)) as well as those of mammals (e. g. rats and guinea pigs, (*Granit* 1941 b)) show such relative differential colour sensitivity of the receptors.

According to *Granit* (1943) intensity perception and colour sense are bound to receptor units of different kinds. The majority of visual nerve fibers give a rela-

*) The most recent investigations (e. g. *Friedrich & Schreiber* (1943)) confirm, however, that *Bohr's* calculations are relatively consistent with the results of practical experiments.

tively wide spectral-sensitivity curve with its maximum at a wave length of 560 μμ corresponding to the curve of the light-adapted eye. The units with wide spectral sensitivity are by Granit called »dominators« and appear to be the organ which produce the impulses determining the perceived brightness of the light. Units with a relatively selective spectral-sensitivity (narrow spectral-sensitivity curve, are found less frequently than the dominators — these units have maximum in the red (580—600 μμ), the green (520—540 μμ), or the blue (450—470 μμ) region of the spectrum. These units, called »modulators« are presumably those which enable the eye to impart to the visual sensation an impression of hue.

Granit's theory of colour perception is, as it appears, a modification of *Young-Helmholtz'* theory but has the advantage of being able to explain why some colour-blind persons can differentiate between differences in intensity also in the colour-blind regions of the spectrum.

C. The Physiological Resolving Power.

1. The Conditions for the Exact Localization of the Image Point in the Monocular Field of Vision.

Even an eye consisting of a spherical surface with sense cells on the concave side and a hole as optic system can to a certain extent orientate two-dimensionally in the visual field provided that the sense cells can perceive the variation of the illumination on the spherical surface, i. e. have a sufficiently good intensity discrimination.

The accuracy with which a sense organ projects the points in the visual field will depend firstly on the optical construction of the organ, especially on the resolving power of the optical system, and secondly on the size of the receptors and the number of independent receptors per unit of area in the sensory neuro-epithelium of the organ. The part played by both factors will be enlarged upon in the following.

a. The influence of the optical system.

The resolving power of an optical system is the term which is used to measure the distinctness of the image attained in an optical instrument, e. g. a telescope or a photographic camera. Because of the diffraction of the light at the edge of the aperture which is a component part of every op-

tical instrument the image of a distant luminous point formed in the focal plane is not a point but a small spot — the diffraction disc (*Airy's* disc) — the light intensity of which decreases from the centre outwards. Concentric with the central maximum are series of alternating light maxima and light minima of gradually fading intensity. The distance from the centre to the first light minimum — generally represented by the letter φ — is only dependent on the wave length of the light used and the diameter of the aperture. As it is assumed, as a matter of convention, that two diffraction discs (*Gullstrand* 1909[375]) can be seen separately, when their relative center distance is φ, φ is said to be the resolving power of the optical system in question. φ is generally given in seconds of arc and is calculated from the formula: $\sin \varphi = 1.22 \frac{\lambda}{2R}$, where λ is the wave length of the light and R the radius of the aperture of the system (*Gullstrand* 1909[375]).

The size of the smallest subjective retinal image imaginable on the retina by the optical system, depends on the optical imperfections of the eye, i. e. the spherical and chromatic aberration, dispersion in the refractive media and also the diffraction of the light at the pupillary edge. The effect of all these factors is that the image of the point on the retina is not a point, but a small light spot with its highest intensity centrally. With diminishing size of pupil the said optical imperfections will decrease while the diffraction phenomena will increase. Consequently there will be one size of pupil at which the resolving power of the optical system is best.

By calculation *A. Gullstrand* (1909[375]) has found the resolving power of the optical system of the eye for yellow or white light to be 49 sec. of arc for 3 mm pupil and 72 sec. of arc for 2 mm pupil without making any allowance for the aberration, however, as this should not be of any essential importance at such a small pupil size. With due allowance for both aberration and diffraction of light *H. Hartridge* (1918) states correspondingly that the optimum resolving power for the optical system of the eye is 44 sec. of arc (3.1 μ) at a pupillary diameter

of 2.5 to 3 mm for yellow or achromatic light. Finally a value of 40 sec. of arc is stated by *Kühl* (1924 [376]).

If the resolving power of an optical system with approximately the same aperture as the eye (2.2 and 3.5 mm) is determined by experiments we shall, according to *Rubin* (1915 [191]), find values of 47 and 28 sec. of arc respectively for an object consisting of two luminous parallel lines.

According to these statements we seem to be justified in assuming that the magnitude of the resolving power of the optical system of the eye is slightly less than 1 minute of arc for luminous points on dark ground.

b. Remarks on the structure of the fovea.

The size of the smallest subjective retinal image imaginable will correspond to the size of one receptor in angular measurement. It will be impossible to recognize a difference in the position of an image within the range of one receptor. For this reason the fineness of the receptor mosaic will be the second of the two chief factors determining the accuracy of the spatial orientation in the field of vision, and it will itself depend on the size of the individual receptors and a possible coupling of the receptors into greater units.

The size of receptors and their number per unit of area.

The receptors in the fovea consist of visual epithelium cells, the so-called cones. The size of the diameter of the central cones in the human fovea as stated by various authors varies somewhat. According to *Østerberg* (1935 [18]) the following values have been tabulated (Table II).

According to *Østerberg's* own countings the diameter of the cones in the foveal centre can be calculated to 2.6 μ. For the cones outside the center the diameter increases with increasing retinal eccentricity. See Table VI p. 109.

The reason why the figures in table II varies considerably may either be ascribed to the histological methods used or be conditioned by individual variations between the eyes examined.

Table II.

The diameter of the cones in the central part of the fovea (According to Østerberg 1935).

Author:	*Year:*	*Diameter in μ:*
Henle	1852	3.0
Kölliker	1852	3.0
M. Schultze	1866	2.5
Koster	1895	4.4—4.6
Greeff	1901	2.5
Heine	1902	4.0
Rochon-Duvigneaud	1906	2.0—2.2
Fritsch	1908	1.8—4.5

It is a matter of course that the shrinkage of the tissue caused by the usual histological hardening will make fine measurements somewhat uncertain and this holds good even if — as *Østerberg* (1935) did it — an attempt is made to control for the shrinkage. It is (according to a verbal statement to the author) for this reason that in his book *Østerberg* (1935) has omitted to calculate the diameter of the cones. The individual variation plays an essential part as well. According to *Fritsch* (*Østerberg* [26]) the diameter of the central cones varies individually from 1.8 to 4.5 μ.

For the reasons given it will be correct to consider all statements with regard to the size of the cones to be only relatively accurate.

The absolute number of cones per unit of area has a secondary importance only in this connection. However *Østerberg* (1935 [84]) found 147300 cones per sq.mm in the foveal centre of the retina examined by him, and *Fritsch* (*Østerberg* [23]) a lower and upper limit of 10000 and 187500 cones per sq.mm respectively.

The centripetally conducting neurones of the fovea.

It is usually assumed (*Chievitz* 1889, *Wilbrand* and *Saenger* 1904 [168], *F. Schieck* 1928 [580], *Kolmer* 1936 [394]) that in the fovea there are one bipolar and one optical ganglion cell with

its neuron for each cone, but it cannot be excluded, however, that in the human fovea there are also polysynaptic bipolars and ganglion cells, as our knowledge of the histology of the human retina is rather small (*Kolmer* 1936 [394]). *Polyak* (1936) has found both mono- and polysynaptic bipolar cells in the fovea of monkeys and apes, however, mostly monosynaptic and the ganglion cells corresponding to these bipolars are usually monosynaptic.

Even if it was known that in the fovea there is one optical ganglion cell for each cone, we should not be justified in concluding that the fibres of the pathway from visual epithelium to the cerebral cortex are usually isolated in their entire length, as it is quite possible to imagine an ascending convergence*) of the fibres in the lower visual centres in the external geniculate body and the superior colliculi. The part played by the transversal retinal connections is also unknown although certain conjectures have been made with regard to the amacrine cells which appear in greater numbers in the fovea than in the periphery and possibly convey efferent inhibitory impulses (*Graham & Granit* 1931) see p. 75).

As very little is thus known about the afferent pathway of the peripheral sense cells in general and as in particular it is not known whether a certain number of cones by means of transversal fibres are connected to the same optical neuron it is judged convenient for the time being to use the term »functional unit« about the number of cones connected to the same independent optical ganglion cell. The size of these units as measured by means of sense-physiological experiments will be discussed later (see p. 23). On a histological basis no more can be said about the size of the functional units than that in the centre of the fovea they consist of at least one cone (diameter about 3 μ or 35 seconds of arc). As the size of the foveal cones outside the centre proper increases the diameter of the functional units will probably also vary.

*) By convergence (a term which originates from *Granit* (1936 [3])) a reduction of the number of nerve fibres is understood, the neurones uniting and becoming fewer, convergence is thus the opposite of arborization.

2. The minimum separabile.

The first attempt at relating the resolving of two points to the structure of the retina is due to *E. H. Weber,* who in 1846 [527] proposed the term »Empfindungskreise« for such small retinal areas each of which is connected to one »elementary nerve«. Weber further set forth the theory that two points cannot be seen separately unless their retinal images are separated by at least one »Empfindungskreis«.

Synonymous with »Empfindungskreis« *Giraud-Teulon* (1879) has adopted the term minimum separabile.

(Pour que le terme qui représente l'arc sous-tendu par l'élément rétinien isolateur, directeur (cône ou bâtonnet), contienne en lui-même sa signification exclusive, nous proposerons d'adopter la qualification de minimum separabile).

In this book it has been decided to express the fineness of the two-dimensional orientation in the visual field by means of the physiological resolving power or the ability of correct recognition of the relative position of two points, and to measure the same by means of the minimum separabile which is defined as the smallest relative angular distance at which two points can be perceived as separate. Admittedly this definition is not the same as *Giraud-Teulon*'s original one as it does not base the min. separabile on the visual angle of the object, but on the angular size of the retinal element, but with this modified definition the min. separabile is considered to be more useful in practice. If it can be proved that in its new meaning the min. separabile under certain conditions expresses the size of the functional units the two definitions will merge. If so desired this min. separabile might in this case be called the min. separable proper while the term min. separabile in a wider sense might be reserved for the cases where min. separabile bears no clear relation to the retinal mosaic.

If the physiological resolving power — as it might be expected — depends on the exact projection of the image only (i. e. on the optical imagery and the size of the functional units) min. separabile would be independent of the size of

the object as long as the image of the object is smaller than the functional units. A variation in the illumination should have no influence either, except that it might affect the size of the image. The reason why according to the generally accepted view the opposite holds good, namely that the size of the min. separabile varies as the size of the object and always decreases with increasing illumination, seem to be that the experiments on which this view is based have been made with objects illuminated from before, so that besides the optical imagery and the anatomical structure of the eye the intensity discrimination also has an influence (conf. p. 11). Only by using luminous points on an absolutely unilluminated background — as done by one author — it will be possible to eliminate the influence of the intensity discrimination.

In order further to substantiate and expound this theory earlier investigations with regard to the minium separabile will be dealt with in the following, and the importance which in the course of time has been ascribed to this function will be specially mentioned.

a. The dependence of minimum separabile on the size of the test object, its relationship to the retinal mosaic and its utility as a measure for the visual acuity.

The smallest visual angle at which two points or lines can be perceived separately was early used as a measure for the visual capacity of the eye, the visual acuity. The first to determine this angle experimentally was *Robert Hooke*, who seems to have made two measurements, one with lines and one with fixed stars. By the first (1674) (*Birch* 1757 [120]) *Hooke* found that the lines could not be seen separately when they were separated by a visual angle of less than one minute of arc. As to the other measurement (1681) (*Pergens* 1906 [55]) it is stated that two stars could not be seen separately when separated by a smaller distance than one minute of arc.

It has later on frequently been attempted to verify *Hooke*'s

experiments, the results have differed, this can, however, no doubt be explained by the varying methods employed, especially with regard to illumination and the selection of test objects.

Aubert (1865[233]) declares that as far as he knows no human being is able to perceive two fixed stars separated by a smaller angular distance than 3 minutes of arc and concludes that *Hooke*'s instruments have been inaccurate. Later authors who have measured min. separabile for point sources on dark background sometimes in light adaptation have all found values of 2.1 to 3.5 minutes of arc (*du Bois Reymond* (1886), *Wertheim* (1887), *Berger* (1936), *van Heuven* (1937) and *Berger & Buchthal* (1938 a)).

All the same *Hooke*'s figure of 1 minute of arc. for the min. separabile for fixed stars is often quoted as correct, (f. inst. by *Helmholtz* (1896[259]), *Sulzer* (1904[561]), *Heine* (1915[105]), *Hartridge* (1918, 1922, 1936[381]), *Einthoven* (1921)). As moreover the min. separabile for objects consisting of slits or bars (both luminous and illuminated from before) amounts to one half to one minute of arc (*du Bois Reymond* (1886), *Helmholtz* (1896[259]), *Hartridge* (1922), *Wilcox* (1936), *van Heuven* (1937)), it is easy to understand why several authors are of the opinion that the min. separabile is the same for points and double lines (f. inst. *Giraud-Teulon* (1879), *Helmholtz* (1896[258]), *Hering* (1899), *Einthoven* (1921), *Dittler* (1932[382])).

The first systematic attempts to decide the dependence of the min. separabile on the size of the object was made by *Aubert* (1865[228]) with white squares on black ground and black squares on white ground both illuminated from before. *Aubert* found the min. separabile to be smaller for black squares than for white, but for both categories he found the min. separabile to decrease uniformly with increasing size of square (see Fig. 1). The results obtained by *Hofmann* (1925[32]) from similar experiments are shown in the same figure. On the basis of these and other experiments first *J. Bjerrum* (1882[3]) and later on *Pergens* (1903), *Wolffberg* (1910), *Roelofs & Bie-*

rens de Haan (1922) *Hofmann* (1925[34]), *Guillery* (1931[763]) and *Wilcox & Purdy* (1933) have drawn the conclusion that there is no min. separabile which is independent of the size of the squares, in such a way that it may be the basis of a measurement of the visual acuity as a function of the size of the functional units in the retina (see p. 19). In analogy

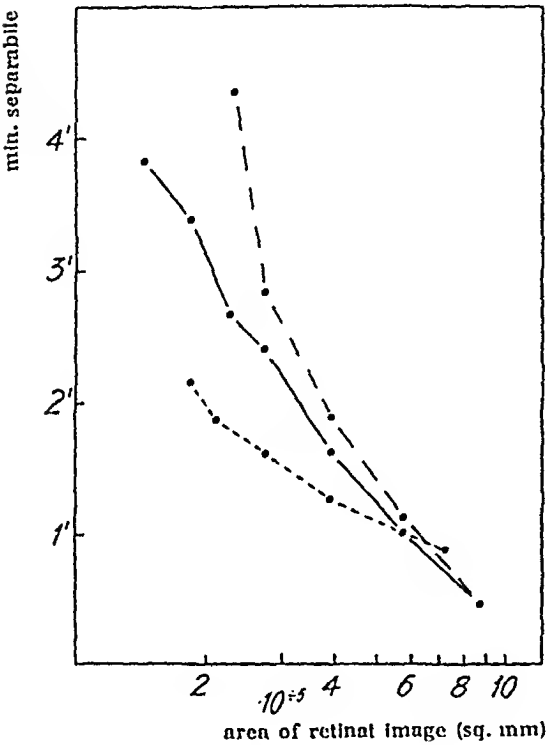


Fig. 1.
The dependence of the min. separabile on size of square, the squares being illuminated from before. Calculated according to *Aubert* (1865[229]) and *Hofmann* (1925[34]).
Abscissa: the area of the retinal image of a square in sq. mm • 10÷5 (log. scale).
Ordinate: Minimum separabile in minutes of arc.
——— White squares on black ground (*Aubert*)
— — — Black squares on white ground (*Aubert*)
– – – – Black dots on white ground (*Hofmann*).

with this these authors hold that all the properties of the min. separabile can be explained with reference to the conditions of visibility of the intermediate area between two adjoining contours i. e. the differential threshold for the enclosed area in relation to the squares. It is thus seen that the condition which must be fulfilled in order that the space between two closely situated diffraction discs (see p. 15) the edges of which cover each other is visible, is that the intermediate space is perceptibly less illuminated than the centres of the diffraction discs. This is consistent with the fact which has often been ascertained that the intensity discrimination decreases when the test area is diminished (see p. 10).

The conception of the min. separabile outlined here must in any case hold for the cases in which the space between two adjoining contours is less illuminated than the two areas separated, but not absolutely unilluminated, or, in other words, the illumination of the intermediate space exceeds the intensity threshold. The theory cannot, however, be considered to be valid in all cases as it does not hold for luminous points on dark ground. When this is nevertheless the generally accepted view (conf. e. g. *Hofmann* 1925[28]) it is due to the assumption that two luminous points can be seen separately when the distance between them is so small that the corresponding diffraction discs partly cover each other on the retina. This, however, is definitely quite wrong (see below).

Moreover it is usually overlooked that the edges of the diffraction discs in case of faint illumination must be assumed to be below the intensity threshold, so that the images perceived have sharp boundaries. For this reason it will be feasible to consider the space between the retinal images of two faintly luminous points as unilluminated, when the points can be recognized as separate, and the min. separabile will then be a function of the size of the functional units.

In support of these considerations which have been propounded by *Berger & Buchthal* (1938 a), it was found by these authors and by *Berger* (1936 and 1939) that the min. separabile under certain experimental conditions is independent of the size of the object. Thus the min. separabile for luminous squares of threshold brightness on absolutely unilluminated background and for light-adapted eye is constant (about 3 minutes of arc, measured as the distance between adjoining sides of the squares) until the retinal image of each square exceeds 18 sq.μ (length of side about 40 seconds of arc) when the min. separabile gradually decreases numerically. The large min. separabile for diminutive squares (»points«) is explained by the authors by assuming that the functional units each consists of a number (3 to 5) of transversely connected receptors

(cones). On account of the involuntary oscillating movements of the eye (for further details see p. 56) the retinal image must fall on various units successively, and the resulting min. separabile must therefore be considered as the resultant of a series of individual observations, and thus give an expression of the average diameter of the functional units, which must be assumed to vary in size.

The diminishing of min. separabile with increasing size of square is explained by the fact that statistically there is a greater chance of enclosing one of the smallest functional units between the adjoining sides of the squares. In conformity with this is was stated by *Berger & Buchthal*'s subjects that when quite close to each other the squares were seen to be contiguous in some places and separate in others, the latter

The min. separabile is, however, stated to be independent of the size of the test object not only in case of luminous squares on unilluminated ground but also in another case. It is thus reported by *Berger* (1939) that for absolutely unilluminated squares on luminous ground of threshold brightness the min. separabile decreases with increasing size of square until it is appr. 1 minute of arc and then it remains constant, a fact which indicates that there is now at least one of the functional units enclosed between the retinal images of the squares. This minimum value of the min. separabile is obtained when the side of the squares is more than 5 minutes of arc.

In case of illumination from before on an otherwise unilluminated ground or in case of paper squares illuminated from before it has not been possible to prove the min. separabile to be independent of the size of square in any range of measurement. *Berger & Buchthal* therefore conclude that the min. separabile for objects illuminated from before gives no indication of the size of the functional units, but only of the intensity discrimination. This explains the considerable difference between the results obtained by previous authors on one side and those of *Berger & Buchthal* on the other.

The theory about the importance of the intensity discrimination in case of objects illuminated from before has also been substantiated by experiments carried out by *Berger & Bøje* (1937) determining the min. separabile at various oxygen tensions in the inspired air. *Berger & Bøje* based their experiments on the assumption that a variation in the oxygen tension of the air would have much more influence on the central nervous system than on the peripheral organs (in this case the retina). The authors further base their work on experiments by *Gellhorn* (1936) according to which the intensity discrimination for light is strongly dependent on the oxygen tension. *Berger & Bøje* proved by their experiments that while a reduction of the oxygen tension had practically no influence on the min. separabile for luminous squares on unilluminated ground, the min. separabile for black squares on white ground illuminated from before increased rapidly under the same conditions. From this the conclusion could be drawn that the extra centrally located factor, which appears in connection with illumination from before, is probably the intensity discrimination.

As *Berger & Buchthal*'s experiments and theories seem to be of fundamental importance for the conception of the min. separabile and in a broader sense for the conception of visual acuity, and as the experiments have apparently not been verified by others, it has been attempted to reproduce the most important experiments (see chapter IV) and to elaborate them on certain points.

b. The dependence of the minimum separabile on the illumination.

As it has already been suggested on page 19 it might be expected that if the min. separabile was merely a function of the resolving power of the optical system of the eye and of the size of the functional units, it would be independent of the illumination. The functional units on which the space between the two images of the object falls will either be illuminated below their intensity threshold (or unilluminated) and consequently inactive or be stimulated and active. If the intermediate

space is not illuminated above its intensity threshold then the min. separabile will be a function of the size of the functional units. If on the other hand the units in the intermediate space are stimulated, the differential threshold for the said units in relation to the units on which the images of the objects fall, will be exceeded when it is possible to recognize the objects as separate. When these facts are taken into consideration it is no wonder that investigations on the dependence of the min. separabile on the illumination have given results which are mutually inconsistent.

Aubert (1865 [230]) who was one of the first to work on the min. separabile found that the min. separabile for squares illuminated from before decreased with increasing illumination. *Aubert* and *Volkmann* (1863 [86]) observed moreover that for fixed stars the opposite holds good. This difference has also been confirmed by later experiments, but the importance of this fact has usually been underrated. In order to render the following representation more perspicuous the min. separabile for objects illuminated from before and for luminous objects on unilluminated ground respectively will be dealt with separately.

Objects illuminated from before.

The most commonly used objects illuminated by borrowed light are squares or figures consisting of bars, black on white ground or vice versa (e. g. *Tobias Mayer* (1754), *Aubert* (1865 [230]), *Helmholtz* (1896 [425]), *Hecht* (1928), *Klenowa* (1935), *Kravkov* (1938)). We shall here leave out of account both the prong-figures used by *König & Uhthoff* (1886) whose experiments are often described as classical (a. o. by *Hecht* 1925) and test types. In both cases not only the part of the object to be resolved varies, but also the size of the whole object. Finally the visual acuity measured by reading letters is such a complicated function that it does not lend itself to this purpose (see p. 49).

It has usually been found for figures illuminated from before that min. separabile decreases when the illumination increases, but increases again when the illumination becomes

so strong as to create a glare. This result must be taken with a certain reservation, as none of the authors have kept the eye of the subject constantly adapted during the experiment, but left the adaptation level to be determined by the illumination of the test object. As the size of the pupil has not been controlled either, it is impossible to estimate the influence of the various variables on the result. If these objections are disregarded, and an attempt is made to explain the course of the curves found, it will be reasonable to adopt the theory, which has already been set forth, to the effect that the min. separabile for objects illuminated from before is a function of the intensity discrimination of the retina.

According to *Weber-Fechner*'s law the just perceptible difference in light intensity is a constant fraction of the total light intensity, or, in other words, the intensity discrimination is constant (see p. 7).

If this law is assumed to hold good then the min. separabile, in the cases where it is an indication of the intensity discrimination of the retinal area enclosed by the squares, should be independent of the intensity (see p. 23). As it has nevertheless often been found that for objects illuminated from before the min. separabile decreases uniformly with increasing illumination, it has been necessary to explain this fact by simultaneous boundary contrast (*Hering* 1925 [157], *Hofmann* 1925 [42]) a subjective, possibly centrally located phenomenon about which experience has taught us that it becomes more manifest with increased illumination, but which at the same time is a phenomenon which it is difficult to measure quantitatively.

The simultaneous contrast cannot, however, be used as an explanation in the present case as — according to *Hering* (1925 [141]) — the phenomenon disappears by careful fixation which is a necessary condition of experiments on the min. separabile.

As an explanation of the difference between the intensity discrimination and the dependence of the visual acuity on intensity it is alleged by *Wright & Granit* (1938 [70]) that the contrast between the test object and the background is greater

when measurements of visual acuity are carried out, than when the differential threshold is determined.

It is, however, doubtful whether such a difference in contrast actually exists. This can be seen from the following:

In order to compare the differential threshold with the min. separabile the former should be determined for a retinal area of the same size as the space between the twin objects used for the determination of the min. separabile, and as the light difference increases considerably (see p. 10) with decreasing size of object, the contrast should increase correspondingly.

Weber-Fechner's law is valid, however, only for light intensities within a certain range, i. e. for a light-adapted eye not exposed to glare, provided that the test object solely determines the state of adaptation and is sufficiently large to do so. For higher and especially lower light intersities the difference in light intensity must constitute a steadily increasing fraction of the total intensity to be recognizable. The intensity discrimination of the eye thus varies with the light intensity of the adaptation field, and for objects illuminated from before it can probably explain the diminution of the min. separabile when the adaptation illumination increases (*Lythgoe* 1932).

Luminous objects on unilluminated ground.

Although there is no obvious reason for considering luminous points to be less suitable test objects than squares or even letters illuminated from before, it has nevertheless up to recent years usually been omitted to investigate the min. separabile for luminous points, and only objects illuminated from before have been used when dealing with the variation of the visual acuity with the light intensity (e. g. *König & Uhthoff* (1886), *Klenowa* (1935), *Anthonisen* (1936), *Kravkov* (1938)). On the basis of such investigations it is therefore often held to be established that the visual acuity always varies in the same way with the illumination whether the visual acuity is measured by the min. separabile by means of objects illuminated from before or by means of luminous objects on

dark ground. Both *Aubert*'s (1865[233]) and *Volkmann*'s (1863[86]) observations of the min. separabile for fixed stars as well as some recent investigations show that this is, however, not the case.

In 1932 it was proved by *Wilcox* that the variation of the min. separabile with the illumination differs according to the test object employed.

While the min. separabile for two dark lines on luminous ground steadily decreased with increasing light intensity of the ground he found that the min. separabile for the reverse object (luminous slits in a dark field) with increasing light intensity decreased only until it reached an optimum of about one minute of arc at medium intensity and after that again increased.

By corresponding experiments on the min. separabile for somewhat smaller objects (squares) *Berger* (1939) — like *Wilcox* — found that the min. separabile for dark squares on luminous ground decreased steadily with increasing intensity of the ground. But for luminous squares on unilluminated ground *Berger & Buchthal* (1938 a) and *Berger* (1939) could not ascertain any reduction of the min. separabile with increased retinal illumination, on the contrary they found a uniform increase of the min. separabile in the whole range from threshold intensity to glare. Corresponding results have been obtained by *van Heuven* (1937) probably with a somewhat smaller range of measurement.

And finally *Hecht & Mintz* (1939) have determined the visibility of an opaque thread on a luminous circular ground (angular diameter about 14°) at various light intensities, in other words, they have determined the min. separabile for two large semi-circular areas. For this object the min. separabile diminished with increased light intensity.

Three different results have thus been obtained from experiments on the min. separabile for two luminous areas separated by a dark stripe. When the method employed is considered more closely it appears, however, that both *Wilcox* and *Hecht* during their experiments have varied at least two experi-

mental conditions at the same time, so that it is difficult to deduce anything from the results. It may thus be held against the method used both by *Wilcox* and *Hecht* that the experiments have not been carried out with constantly light-adapted eye. Both authors leave the state of adaptation — as *König & Uhthoff* (1886), *Kravkov* (1933 and 1938), *Klenowa* (1935) and *Shlaer* (1937) — to depend on the light intensity of the test object, i. e. they vary the adaptation level of the eye and the light intensity of the test object simultaneously during the experiments. For this reason a quantitative estimate of the influence of the latter variable cannot be made.

Hecht & Mintz do not use an »artificial pupil« and justifies this by referring to investigations by *Berger & Buchthal* (1938 a) which are supposed to prove that the min. separabile is independent of the diameter of the pupil if only this is above 1 mm. This, however, is not correct. *Berger & Buchthal* have certainly proved this fact, but only for threshold brightness or constant illumination of the retina, while for constant intensity of the test object the min. separabile diminishes rapidly with reduced pupillary size (reduced retinal illumination). It is therefore probable that with *Hecht*'s apparatus there is a simultaneous variation of the adaptation level, the light intensity of the test object and the pupillary size, without it being possible to distinguish the influence of one factor on the experimental results from that of the others.

In their discussion of *Wilcox's* experiments *Hecht & Wald* (1933—34) attempt to deny that *Wilcox* has investigated the visual acuity through his experiments with luminous slits as the intensity of retinal images of such small dimensions (angular measurement 20' by 2'22") cannot be considered to be expressive of the retinal illumination as a whole, for which reason the function investigated by *Wilcox* is merely assumed to be a phenomenon caused by glare. This argument does not, however, hold, as it has never been ascertained that the image of a test object for determining the visual acuity must cover a large part of the retina. On the contrary it is usually con-

sidered a matter of course to use objects the images of which fall within the limits of the fovea.

It appears for the above reasons that neither *Hecht*'s nor *Wilcox's* results are directly comparable with those of *Berger & Buchthal,* as the experiments made by the latter were carried out with constant light adaptation and with an »artificial pupil«. It cannot be taken for granted, however, that the above facts alone can account for the different results, as possibly the highly different visual angles of the test objects employed may be of importance. *(Berger & Buchthal's* test object was luminous squares of 40" by 40" or 64' by 64', *Wilcox* used luminous slits, the dimensions of which were 20' by 2'20", and *Hecht's* object was an opaque thread on luminous ground of 14°).

The test objects used by both *Wilcox* and *Hecht & Mintz* are so large that the min, separabile approaches to or is smaller than the resolving power of the optical system of the eye (almost 1'). In that case the min. separabile can only be a function of the intensity discrimination, and the curve represents the dependence of the differential threshold on the illumination. The smallest min. separabile found by *Hecht & Mintz* is 0.5 seconds of arc, a value corresponding to a calculated differential threshold of 1.17 per cent.

As it appears from the foregoing it is not possible to base anything on the results obtained by *Wilcox* and *Hecht & Mintz,* consequently we have only *Berger & Buchthal's* experiments from which conclusions can be drawn. On a first consideration of these it seems as if the results of the experiments are at variance with the theory that for luminous squares on unilluminated ground the min. separabile is independent of the intensity of the light. The dependence found is, however, easily explained as being caused by the change in size of the image of the square which is due to the dispersion of light in the eye. The geometrical-optical path of the rays are the same at low and high light intensities, but it is a well known fact that when the light passes through refractive media a dispersion takes place *(Hering* 1925,[141]) so that the contours of the images become less definite and, in case of

high light intensity, the boundary lines between illuminated and unilluminated retinal areas will be displaced reducing the size of the unilluminated areas, so that the apparent size of luminous objects is increased. This dispersion of the light is more pronounced the more darkadapted the eye is. This fact is usually explained by means of the phenomenon »simultaneous boundary contrast«, which according to experience is most pronounced during light adaptation, and so to speak maintains a constant »fight« with the dispersion of the light — the explanation is not quite sufficient however.

The dispersion of the light can on the whole explain the results obtained by *Berger & Buchthal* and *Berger* both for luminous and unilluminated squares in the following manner. When the light intensity is increased the boundary between the images of the squares and the intermediate space will be displaced in the direction of the unilluminated retinal areas. Thus the unilluminated gap between the luminous squares will become narrower, and the distance between the squares must be increased if they are to appear discrete. The opposite holds for unilluminated squares on luminous ground, so that in both cases the absolute width of the retinal intermediate space which conditions the recognition of the min. separabile is independent of the intensity of the light. *(Berger & Buchthal* 1938 a, *Berger* 1939). This is in confirmity with the theory that the min. separabile in this case is a function of the size of the functional units.

Also *Wilcox* (1932, 1936) explains his results by means of the dispersion of the light in the eye, but, as it was shown by *Kravkov* (1933), the theory does not seem to agree with the result obtained by *Wilcox* that the min. separabile for two luminous slits with growing light intensity first decreases and then increases.

Berger & Buchthal (1938 a) explain this discrepancy by assuming that during *Wilcox'* experiments the back ground of the slits have not been absolutely unilluminated.

In the foregoing the influence of the intensity discrimination and the dispersion of the light in the refractive media on the

min. separabile with varying illumination, has been discussed. Theoretically a third factor could be of importance: an alteration in size of the functional units or their number per unit of area conditioned by the intensity.

Thus *Broca* (1901) has propounded the theory that when the illumination is increased the outer segments of the cones is compressed by migrating pigment. Assuming that only the outer segments of the cones are light-sensitive then the images of two point sources would be able to approach each other more on the retina without stimulating the intermediate unilluminated cone. However, *Broca* seems to have overlooked the fact that not only the diameter of the individual receptor cell but also the intermediate substance must be included when speaking of the size of the receptor units, so that the decisive figure is the number per unit of area or per unit of length.

In 1925 *Hecht* advanced another theory which was intended to explain the dependence of the intensity discrimination and consequently of the visual acuity on the intensity. *Hecht* assumes that the individual sensitivity of the foveal cones within the same area varies considerably, according to the normal law of variation. For each perceptible increase in the illumination a further number of cones will be stimulated above their individual threshold and start sending impulses centripetally, and with increased illumination all the cones will thus gradually go into action. Hereby the number of active units per unit of area will increase and the min. separabile decrease.

This theory has been repeated in a number of *Hecht's* later papers (1928, *Hecht & Wolf* 1929, *Hecht* 1930 and 1931, *Hecht & Wald* 1934, *Hecht,* 1935, 1936) and has caused some discussion. It is thus maintained by *Wilcox & Purdy* (1933) that dark spots corresponding to inactive units are never seen on a luminous ground, and that a dark point on luminous ground is always perceived as circular (ref. however pp. 178 and 179). It is further stated by *Wright & Granit* (1938, [67]) that *Hecht's* theory does not take into account the rise in frequency of the responses from the cones already stimulated, and that

it requires a considerable difference between the individual thresholds of the cones, which is not in conformity with the results obtained from electro-physiological experiments. And finally it must be assumed that the neurones inserted between the cones and the cerebral cortex adjust differences in intensity thresholds, if any.

While *Hecht* seems to assume that the intensity threshold of the individual unit is constant, but varies considerably from one unit to the next, several authors *(Bartley* 1937 and 1939, *Berger & Buchthal* 1938 b, *Granit* 1941 a) have lately pointed out that the individual unit is probably the seat of a spontaneous alternating or rotating activity consisting of one active, one absolutely and one relatively refractory period.

(In one of his later papers (1939) *Hecht* certainly states that he has always imagined this possibility, but from a previous paper *(Hecht & Wolf* 1929) the following may be quoted. »We frankly incline towards the fixed threshold interpretation, largely because it seems unlikely to us that a given cell can vary in its threshold from moment to moment over sueh a very large range of illumination«.)

The first authors to prove the alternating activity of the retinal units were *Granit & Therman* (1935), who found that when the retina was stimulated by intermittent light only every second flash of light would — under certain conditions — cause fluctuations of potential in the electro-retinogram. Any further explanation of the phenomenon was not given. In 1937 *Granit & Munsterhjelm* supplemented the observation with new experiments and were now able to prove the alternating activity of the units, which they termed »switchboard effect«.

(»The switchboard effeet shows that there is a number of alternative pathways or eouplings of pathways from the entranee station in the receptor to the optic nerve. This eonclusion is independent of whether the switehboard effect is a normal or a pathologieal process. The meehanism whereby sometimes one, sometimes another circuit is »switched on« can hardly be localized elsewhere than in the neurones between the receptor and the optic nerve. It is, of course, not neeessary to represent the functional mosaic of different types of nerve responses or electrical component potentials or a pattern of stable units with different properties. The seeret of the problem

may lie in the switchboard effect being itself capable of providing different types of responses by suitably coupling units differing very little from one another.«)

In the same year (1937) and later on in 1939 *H. S. Bartley* also published some electro-physiological experiments by means of which phenomena, that could only be explained by an alternating function of parallel units in the stimulated retinal area, could be traced. *Bartley* therefore assumed that for a certain retinal area there must be multiple pathways from the sensory cells to the cortex, in any case for part of the way.

The first to correlate the theory of the alternating activity of the retinal units with results of sense-physiological measurements were *Berger & Buchthal* (1938 b). Experiments on the form sense proved that in order to perceive the angularity of a polygon it was necessary that the retinal image (with threshold illumination) fell on about three times as many retinal units as calculated (see p. 41). (According to *Berger & Buchthal's* previous experiments (1938 a) on the min. separabile each unit was assumed to consist of 2—3 cones).

This could only be explained by the assumption that at any time only about one third of the units stimulated were active while the remainder were refractory. It must be assumed that the individual unit while being continously stimulated alternates between a state of responsiveness and a refractory period. As it was also found that the form sense improved with increasing light intensity of the test object, whether this was luminous on an unilluminated ground or the opposite, *Berger & Buchthal* were of the opinion that this dependence could best be explained by assuming that with an increased illumination the units are stimulated earlier in their relatively refractory period so that more units per unit of time and area are active.

These authors did not employ this theory as an explanation of the dependence of the min. separabile on the intensity, as all the phenomena shown could be explained solely by the dispersion of the light in the refractive media, with two exceptions, however.

One exception is due to the observation that the min. sepa-

rabile for luminous squares on unilluminated ground increased with increasing intensity at a rate which was higher than the one at which the min. separabile for unilluminated squares on luminous ground decreased. This was explained by the assumption that the aberration phenomena set up by the refractive media and their imperfections are less visible with unilluminated squares than with luminous squares, because the larger luminous surface of the former would give a stronger scattered illumination of the retina in relation to which the less luminous aberration phenomena would recede. In conformity with this it was found that the min. separabile for large luminous squares did not increase so much with increasing illumination as the min. separabile for small squares.

The other exception is constituted by the fact that the min. separabile for luminous as well as for unilluminated squares decreases if a luminous frame is placed round the luminous squares at some distance from them or if the size of the luminous ground on which the dark squares are placed is increased. The only explanation of this strange phenomenon which *Berger* (1939) could offer was to suggest a possible »variation in the sensitivity of the organ as a whole«.

As, however, the min. separabile with *Berger's* experimental method is supposed to be independent of variations in the intensity discrimination of the eye (vide p. 25), it would be more natural to assume that the phenomenon in question is due to the fact that the illumination of a retinal area may cause a shortening — provoked by a peripherally or centrally located process — of the refractory period of the functional units over a larger field. (conf. electro-physiological experiments on interaction between separated areas e. g. *Graham & Granit* 1931).

3. Perception of Movement.

It is mentioned on page 5 that under suitable conditions the perception of movement will be a function of the physiological resolving power. This is rendered quite obvious by a closer study of the simplest conditions of perception of movement by a visual organ. While a single sensory cell will be

unable to record the movement of a constant light source in the visual field, and only by means of successive relative light impressions be able to give any information with regard to the distance of the light source, a sensory organ consisting of only two sensory cells will be able to record a movement when the cells are successively stimulated, just as they — as previously mentioned — would be able to impart to the individual a certain one-dimensional spatial orientation. Only when the visual organ consists of a larger number of units distributed over a surface will it be able to perceive the position of an object in two dimensions and it will be possible to determine whether the object is at rest or moving in a plane perpendicular to the visual axis.

The condition which must be fulfilled in order that the movement of a moving object is not observed is that all stimulated units are being constantly stimulated. If the movements of the image of the object are so small that no units are stimulated above their successive differential thresholds it will be impossible to perceive the movement. As the successive intensity discrimination depends on the speed with which the intensity varies, a movement may be so slow that it will be impossible to recognize it; just as an oscillating movement in case of sufficiently high frequency may give a constant immobile image. If we imagine a point image to be oscillating with medium frequency*) on a surface of receptor units, the movement will not be recorded as long as the whole movement takes place within the same unit. Perception will only be possible when the excursion has reached such a size that the image during its movement strikes a neighbouring unit so that this is stimulated above its differential threshold. If the image corresponds to a luminous point of threshold brightness on unilluminated ground then its movement will only be observed when a neighbouring unit is sti-

*) According to *Stratton* (1900) and *Basler* (1908) the frequency has no influence on the perception of movement when it is kept within certain limits (about 1½—3 times to and fro per second). In case of lower frequency it is difficult to observe the movement and when the frequency is above 3.5 flicker will arise and disturb the observation.

mulated above its intensity threshold. At the same time as the neighbouring unit is illuminated the image will leave the unit originally stimulated, so that this will be stimulated below its threshold. The movement must thus be of the same magnitude as the diameter of one functional unit in order to be recognizable.

If these theoretical considerations are to be applied to human vision a certain regard must be paid to the involuntary movements of the eye. Even with the most careful fixation the eye will perform both small oscillating movements over an angle of the same magnitude as a cone and with a frequency of about 70 per second, and also some less frequent, larger excursions, displacing the centre of the image on the retina (see p. 56).

It should be expected that at any rate the latter larger movements would give a subjective impression of movement. It is quite probable, but so far not proved, that this explains the fact that a carefully fixated luminous point on unilluminated ground often appears to be moving.

If an attempt shall be made at calculating the size of the oscillating movement which must be performed by a luminous point of threshold brightness for the movement to be perceptible with central vision, it is necessary to know the oscillation of the eye (about 30 seconds of arc in both directions from the start position), the diameter of the retinal image, which can be estimated at about 60 seconds of arc (conf. p. 56), and the diameter of the functional units which, according to *Berger & Buchthal* (1938 a), may be estimated at 200 seconds of arc. According to this the retinal image will on account of the oscillation of the eye travel over a total distance of 120 seconds of arc so that an additional movement of the object of above 80 seconds of arc will have the effect that the image on an average stimulates more than one functional unit, so that the frequency of the movement of the object becomes recognizable. In case of a somewhat smaller excursion it may be possible to observe movement, but not of the same frequency. Not until the movement of the image is still smaller will the image seem definitely immobile.

Of experimental results fairly comparable with the above calculation only one, published by *Basler* (1908), is found in the literature. The object employed is a luminous square (52 by 52 sec. of arc) on unilluminated ground. For two subjects the smallest movement which can be recognized is stated to be 75 and 115 sec. of arc respectively with central vision, which is sufficiently consistent with the calculated value. As *Basler's* experiments were performed with dark-adapted eye and consequently with uncertain fixation some similar experiments but in reliable light adaptation have been carried out in order to test the validity of the calculations given above (vide chapter V).

For larger objects (a luminous slit, width 52" by 8'40" length) *Basler* states the threshold for visible movements to be less than for a luminous point (60").

If the surrounds of the object are not homogenous or unilluminated, but give rise to successive estimation of distances between the object and details of the surroundings, even smaller movements can be perceived (5" *Stratton* (1902), 20" *Basler* (1906)). In this case it is quite impossible to analyse the theoretical basis, and this is even more true if the moving object does not maintain its distance from the eye, but approaches or recedes and thereby alters its visual angle. As the thresholds are so low that they cannot be compared with the size of the functional units the intensity discrimination must presumably have some influence.

As a special instance of perception of movement it may be mentioned that one-eyed persons by means of the »monocular stereoscopic vision« are able to train their ability to estimate distances so well that they are able f. inst. to play lawn tennis. It must be assumed that one of the essential reasons why this is possible, is the conception — acquired by practice — of the apparent size of the moving object (the tennis ball) at various distances from the eye. Among other important factors the movement of the ball in relation to the background (the parallax) and the sensation of accommodation may be mentioned.

4. Form Sense.

The form sense is here defined as the ability to recognize the primitive contours of objects in the monocular visual field.

When using this definition as a basis it must be considered unfortunate to use the term form sense as synonymous with the visual acuity measured by reading letters as *Hofmann* (1925, [19], [27]) or with visual acuity generally as it is done f. inst. by *Dobrowolsky & Gaine* (1876 a), *Bjerrum* (1882, [1]), *Adler* (1934), *Duke-Elder* (1938, [1194]).

When examining the relation between the form sense and the retinal mosaic it will be natural to determine the smallest retinal image allowing perception of form. Any object seen under a minimum visual angle will be seen as a »point«. Edges and prominences — if any — will only be seen under a larger visual angle. The simplest way of determining the form sense will thus be to determine the minimum visual angle at which a polygon could just be perceived to be angular or the maximum angle at which it is still perceived as circular. The number of functional units which the image of a regular polygon must cover for the figure to be seen as a polygon cannot be seen immediately, but it is obvious — as it was also pointed out by *Berger & Buchthal* (1938 b) — that two angles must not fall on the same unit.

It is in any case certain that perception of form must be impossible if the polygon is smaller, but the condition with regard to the size of the polygon stipulated by *Berger & Buchthal* is hardly sufficient, as the observation of the angularity of the figure will be uncertain. It can thus hardly be imagined that it is possible to recognize the difference between a triangle and a circular figure, both stimulating three units. The circle would probably now and then take on an irregular angular shape (conf. p. 178) but it would not be possible to determine whether the figure was angular until the side of the polygon was of such a length that its mid-point could be seen to be on the same line as the adjoining angles. In order to fulfill this condition the side of the polygon must be $\geqq 2$ times the min. separabile for luminous points on unilluminated ground (i. e. $\geqq 2$ times the average diameter of the functional units).

According to this the form sense should be placed in the system as a measurement of the physiological resolving power, with one reservation, however. Another factor besides the length of the sides of the polygon is the number of sides or ratio between the magnitude of the angles of the polygon and the length of the adjoining sides *(Guillery* 1899); this is especially true of many-sided polygons, and the importance of this factor is difficult to estimate in advance.

By experiments with luminous polygons on unilluminated ground or vice versa *Berger & Buchthal* (1938 b) found that in order to make it possible to recognize the angularity of the figure at threshold brightness the retinal image of the polygon must cover about 3 times as many functional units as calculated (if each angle were to fall on a separate unit). The explanation is to be found (as mentioned on p. 35) in the assumption of an alternating activity of the units, so that only one third of these are responsive at the same time. In the same paper it is stated that the form sense is better in case the light intensity of the test object is high than if it is low, both for luminous objects on unilluminated ground and vice versa, probably because the units are stimulated at an earlier stage of the relatively refractory period in case of high intensity.

In order to estimate the importance which can be assigned to the theory about the alternating activity of the functional units if the side of the polygon must be $\geqq 2$ times the min. separabile, the size of the retinal image could be calculated from the experimental results obtained by *Berger & Buchthal.* If a square is to appear as an angular figure its length of side at threshold brightness must be 7.9 min. of arc (39 μ) for a luminous square and 7.6 min. of arc (37 μ) for an unilluminated square on luminous ground. As the min. separabile (luminous points on unilluminated ground) for the same subjects *(Berger & Buchthal* 1938 a) is about 3 min. of arc, the value of the length of side is thus about 1.7 min. of arc larger than the calculated minimum value. It is thus impossible to thoroughly explain the results except by means of the theory about alternating acticity, but they should be verified by new experiments (vide chapter VI).

D. Complex Functions.

In the foregoing an analysis of the sense of vision into its main components has been carried out, the relations between a series of relatively simple partial functions having been established. Besides these there are three functions: the aligning power, the ability to discriminate thickness of line and the visual acuity measured by recognition of letters, which, because of their complicated nature, so to speak »originates« from several functions. These complex functions are often used when measuring the visual acuity*) both for central and indirect vision, as they are generally considered good indications of this. On account of their wide theoretical and practical importance they will be dealt with in detail in the following.

1. Aligning Power.

By the aligning power (Unterschiedsempfindlichkeit für Lagen, Raumschwelle *(Hofmann* 1925 [55, 58])) we understand the ability to recognize whether two lines are in alignment — just as when reading a vernier.

The aligning power can be indicated by the reciprocal value of the minimum displacement of the lines which can be recognized — »the displacement threshold« expressed in visual angle. According to all available investigations *(Hofmann* 1925 [55]) the displacement threshold is much smaller than the min. separabile, values from 10"—12" *(Wülfing* 1892) down to 5" *(Stratton* 1902) being given for it, and with regard to magnitude it is only comparable to the threshold for just perceptible movement when using a large test object illuminated from before.

As a physiological basis of the displacement threshold *Wülfing* assumed that the contours had to be displaced as much as the width of one functional unit. This theory was repudiated by *Hering* (1899) and *Hofmann* (1925 [58 ff.]) who — basing his theory on a schematic retina consisting of regular

*) here taken to mean: »the accuracy of spatial observation in the plane visual field« *(Guillery* 1931 [761]).

hexagons — assumed that the condition which must be fulfilled for a displacement to be recognizable, was that the outer parts of some new cones were stimulated. This could explain the visibility of a displacement which is a fraction of the width of a cone. The fact that equally large displacements could occur on the same schematical retina without stimulating new cones was explained away by stating that such cases — on account of the involuntary movements of the eye — would only be temporary.

With our present knowledge of the involuntary oscillating movements of the eye (vide p. 56) the conception outlined is too schematic as the frequency of the oscillations of the eye is about 70 per second and the excursion about 30 sec. of arc from the mean position, so that the perceived image must always be considered to be the average of a continuous series of instantaneous images.

Instead of the earlier theories about the physiological basis of the displacement threshold the following may be stated:

When two contours are in alignment the boundary line between illuminated and unilluminated retina falls on one long row of cones.

When one contour is displaced one half of the row of cones will be exposed to an illumination different from that of the other half part, and when this difference exceeds the threshold of the intensity discrimination it will be possible to recognize the displacement.*) The two halves of the row of cones in question may f. inst. be compared to two long and very narrow photometer-fields adjoining each other endwise. According to this the aligning power should be a function of the simultaneous intensity discrimination of the eye. There are, however, two reasons why the aligning pover is not — like the intensity discrimination — independent of the fineness of the receptor mosaic. Firstly the form sense is a necessary condition of the aligning power, as each of the two contours must be recognized as being oblong before there can be any question of

*) According to *Hartridge* (1922) a displacement of the contours of 11 sec. of arc will alter the illumination of one half of the row of cones by about 32 per cent.

aligning power. When the contours are sufficiently short the smallest perceptible displacement will be equal to the min. separabile if the test object is a slit or line. Secondly it is obvious that for the same discrimination factor (in per cent) (vide p. 6) the displacement threshold will be smaller for small functional units than for large ones.

2. Discrimination of Thickness of Line.

When two slits or lines are in alignment and the width of one of them is altered the smallest perceptible increase or decrease of thickness can be used as a measurement of the ability of the eye to discriminate thickness of line. The discrimination of thickness of line has been used as a measurement of the visual acuity by *Adler & Meyer* (1935) (vide table XV p. 169). However, it will hardly be fortunate to use this function as a general measurement of the visual acuity, as it merely concerns a single aspect of the ability of the eye to perceive details in the visual field.

The physiological basis of the recognition of an increase in thickness must presumably be, that the eye on comparing the two half parts of the slit, without judging the absolute thickness or the displacement of the contours, determines which is the b r i g h t e s t.

This assumption is confirmed by the observation that, if the light intensity of one half of the luminous slit is reduced, the said half part will seem narrower than the other. In this connection it may further be mentioned that according to *Rubin* (1915, [186]) the width of »lines without thickness«*) (abt. 30 sec. of arc wide) can only be estimated by the varying »greyness« of the lines.

This means that discrimination of thickness of line, just as the aligning power, is a function of the simultaneous intensity discrimination, and just as it is the case with the aligning

*) a »line without thickness« is a psychological term for a line the thickness of which is not perceptible, a line so delicate that it is not possible alternatively to fixate the right and left border of the line.

power the oblong shape of the slits must be recognizable, the form sense must thus be fulfilled, in order to render a reading possible. But in contradistinction to the aligning power the discrimination of the width of the slit will be independent of the fineness of the retinal mosaic, as the threshold read is not the contour displacement, but a direct comparison between two light intensities. Whether the view outlined here can hold will be examined further in chapter VIII.

3. Visual Acuity as Measured by Recognition of Letters.

By this we understand the ability to recognize and read letters correctly (in the following called by its other name: the minimum legibile).

As nowadays visual acuity is always measured clinically by the ability to read letters or similar figures, it would be natural to define the clinical visual acuity as being identical with the min. legibile. In physiological and ophthalmological text-books the visual acuity is nevertheless often defined as the ability to recognize two points as separated, with the addition that this ability is measured by reading letters (*Heine* (1915 [105]), *Hartridge* (1936 [381]), *J. W. Nordenson* (1936 [23]), *Olmsted* (1938 [208]), *Ronne* (1940 [9])). It is thus assumed that the min. legibile is a function of the min. separabile and consequently of the magnitude of the retinal units, and this is the conception which was accepted by the *French Ophthalmological Society* in 1904.

In the course of time numerous objections — all to the effect that the physiological basis of the min. legibile is rather complicated — has been raised against this view.

Already *Snellen* (1874 [3]) emphasizes, that the unit, 1 minut of arc, suggested by him and on the basis of which his test types are designed, has been chosen arbitrarily. The sole justification for this choice is that letters the details of which are 1 min. of arc wide and the total size of which are 5 by 5 min. of arc can be read correctly by most normal eyes. Thus *Snellen* did not consider the min. separabile to be the basis of the min. legibile, as it is stated by *Adler* (1934).

It is also pointed out by *Carl Hess* (in the report from the International Committee on Uniform Determination of Visual Acuity 1909) that the visual acuity defined as the ability to recognize the separateness of two points, can only be examined by means of test objects consisting of two points, while the visual acuity as measured by recognition of letters must be measured by means of suitable test types, because the reading of letters is conditioned not only by the min. separabile but also by other factors, especially the aligning power (die Beurtheilung von Lageverschiedenheiten). It is therefore impossible to state the influence exercised by light sense, space sense and form sense respectively on the recognition of test types. When nevertheless the min. legibile should not be rejected as an indication of the visual acuity in practice, it is because of its great clinical applicability, but it is necessary to realize that by means of this method something else is measured than by means of two points *(Hess)*.

Although *Hess* rejects the min. separabile as the sole basis of the min. legibile, he states, however, that experience shows that two points can be recognized as separate when the distance between them is one minute of arc.

If this was the case it would not be necessary to assume that the aligning power had any influence on the reading of letters with details subtending 1 minute of arc. It appears, however, from experiments made by *Aubert* (1865 [228]) and *Hofmann* (1925 [32]) that the min. separabile for black dots or squares with a length of side (diameter) of 1 minute of arc on white ground and illuminated from before is about 3 min. of arc and. 4.5 min. of arc respectively when the illumination is good.

As the min. legibile is thus better than it would be if based on the min. separabile only, *Hess's* idea with regard to the importance of the aligning power would seem justified. As it will appear from page 48 the aligning power does not, however, seem to play any part in this connection.

Even in case of a simple figure like *Landolt's* broken ring the reading of the position of the gap does not seem to be conditioned by the min. separabile for the ends of the ring.

If this was the case the part of the ring opposite the gap would be of no importance, which is not consistent with actual facts. On the contrary *Pergens* (1901 and 1902) and *Guillery* (1905) have shown by experiments that e. g. the width of the ring at this place is of great importance for the reading of the figure. It must consequently be assumed that the subjective criterion for the position of the gap is the fact that the circle is brightest here, that is, a direct dependence on the intensity discrimination.

When it is a question of reading letters of the alphabet it is even more difficult to analyze the conditions of legibility. It is a well known fact that the legibility of the various letters in the same line on *Snellen's* test chart is very different. Some letters e. g. V, A and L have such a characteristic shape, that they can be read at a greater distance than others as P and F, R and B. This difference in the legibility of the letters within one line is considered undesirable by many, who propose test charts with equally legible figures, symbols or letters (*C. Hess* 1909, *J. W. Nordenson* 1942).

This particular difference in the legibility may, however, be an advantage, as the transition to the reading of smaller letters — which otherwise often requires a certain pressure on the part of the examiner — is made easier for the subject.

H. Rønne (1940, [9]) emphasizes the fact that the letter which is most difficult to read in each line of *Snellen's* test chart has the same legibility as the easiest letter in the following line, so that the physiological distance between all the lines is the same, and the distance between the lines corresponds to the experimental error.

If it is preferred that the letters in the same line are of different legibility, then easy and difficult letters must occur equally often in the same line and all lines must have the same number of letters, which is never the case on the usual charts.

As anybody, who is experienced in determinations of the min. legibile on patients will know, the min. legibile is clearly dependent on the intelligence of the patient and the training he has in this examination, probably also on his ability and

inclination to make guesses. It is a very important quality of objects consisting in letters and figures that the correct recognition is very much dependent on the »recollected image« (i. e. the psychological after-effects of letters seen previously). Thus the letter A will be recognized by means of its special shape even if it is so small that the cross bar cannot be seen clearly. If the subject knows that he may possibly be confronted with the Greek letter A, then the letter must be larger in order to render the recognition certain. Or expressed otherwise: When investigating the min. separabile the subject is told to register the point at which the two squares are clearly seen as separate, but when determining the min. legibile he is not required to be quite certain of the existence of the cross bar before he registers the letter A. The same directions will be given to the subject when using Landolt's broken ring as when determining the min. separabile. Consequently experiments with *Landolt's* broken ring cannot be considered equivalent to determinations of the min. legibile proper.

The influence of the »recollected image« was investigated experimentally by *Kreiker* (1923) who found that the min. legibile was 130 per cent better when the subject was confronted with a known letter than when he was faced with an unknown figure designed on the same optometrical principles. If a comparison can be ventured between *Kreiker's* results — according to which the visual angle of the details of the figures must subtend 2.3 min. of arc to render it possible to resolve the figures — and the values found by *Aubert* and *Hofmann* for the min. separabile (about 3 min. of arc for squares subtending an angle of 1 min. of arc and 1 min. of arc for squares with the double length of side, vide fig. 1) it will be seen that the recognition of unknown complicated figures can very well be explained on a basis of the min. separabile. It thus seems unnecessary to assume any influence on the part of the aligning power — it would also be rather difficult to account for this influence with regard to the individual letters.

It appears from the foregoing that the min. legibile depends on one or more of the following factors: the min. separabile, the intensity discrimination, the form sense and the »recollec-

ted image« without it being possible to analyze the influence exercised by the individual factors with regard to the various letters. On account of its complexity the min. legibile will consequently hardly be applicable for scientific experiments with regard to the structure and function of the retina. Even the application of unilluminated letters on luminous ground will not — as for the min. separabile — make the matter more perspicuous. It cannot be denied that clinically the min. legibile is indispensable, but it must be realized that the min. legibile is a very complex function, which is perhaps an advantage in the clinic, and that any alteration of the conditions of the experiments may give incalculable results.

It would be very desirable, therefore, if standard conditions could be introduced for the clinical determination of the min. legibile. In the course of time several attempts has been made e.g. by the International Ophthalmological Congress at Naples in 1909 and by *J. W. Nordenson* (1942) without eliciting any unanimous response, however.

Consequently it is necessary to make certain reservations when comparing the values for the visual acuity obtained by different authors.

There are several uncertain factors connected with the clinical determination of the visual acuity which has not yet been mentioned. In the first place the size of the letters on individual commercial specimens of *Snellen's* test chart varies up to 10 per cent. Secondly the white ground of the table will gradually grow yellowish, so that the contrast is diminished and the chart must be discarded. Thirdly the illumination varies to some extent. Some use daylight, others artificial light from before, others again illuminate the chart from behind. Theoretically the patient's distance from the chart is not without importance as the illumination of the retinal image increases when the distance is reduced. Further the min. separabile and probably also the min. legibile improve somewhat with an increase of the size of the ground *(Berger* 1939). The influence of this factors is presumably so small, however, that it has no practical importance. Fourthly there is some variation in the demands

of the individual examiners to the number of letters, which the patient must be able to read in order to have the visual acuity in question. Some (e.g. *J. W. Nordenson* 1942) demand the patient to read all the letters quickly and correctly, others are satisfied when the patients can read the majority of the letters in the line in question. And finally it must be remembered that sufficiently large letters are so large that the retinal image covers more than the field of central vision (vide p. 108), so that the vision measured is certainly foveal vision but not exclusively central vision.

Chapter II

INDIRECT VISION

In the preceding chapter the visual sense with regard to central vision has been analyzed, and a working basis has been created according to which it should be possible to arrange previous investigations on indirect vision systematically in groups. Before doing this, it will be practical to give an account of the fundamental principles — optical, anatomical and psychological — of indirect vision. When the principles of indirect vision as well as previous investigations on indirect vision has been reviewed we shall have formed a basis for a discussion of the validity of the theories, which has been propounded in order to explain the characteristic features of indirect vision.

A. The Principles of Indirect Vision.

1. The Optical Imagery by Indirect Vision.

Only a few authors have investigated the imagery on the eccentric parts of the retina with objective methods.

E. H. Weber (1846 [531]) and later on *Landolt & Nuel* (1873) have by direct observation of the eyes of albinotic rabbits found that distinct images were formed at eccentricities up to 70°. This was possible because the focal distance and consequently the size of the image decreases with the obliquity of the rays through the refractive system of the eye *(Landolt & Nuel,* 1873).

The only objective investigations of the refraction of the various parts of the human retina were carried out by *Ferree, Rand & Hardy* (1931) and *Ferree & Rand* (1933). The expe-

Table III.

Refraction measured with refractometer at various places in the visual field, according to Ferree, Rand & Hardy, 1931.

Type A. (Average for 6 eyes).

Eccentricity	Temporally		Nasally	
	horizontal meridian	vertical meridian	horizontal meridian	vertical meridian
0	+0.25	÷0.08	+0.25	÷0.08
10°	+0.10	÷0.04	÷0.19	÷0.13
20°	÷0.31	+0.35	÷1.08	÷0.06
30°	÷1.27	+0.42	÷1.94	+0.19
40°	÷2.35	+0.67	÷3.23	+0.94
50°	÷3.14	+1.35	÷4.10	+2.31

Type B. (Average for 5 eyes).

Eccentricity	Temporally		Nasally	
	horizontal meridian	vertical meridian	horizontal meridian	vertical meridian
0	+0.30	+0.25	+0.30	+0.25
10°	+0.44	+0.32	+0.23	+0.20
20°	+0.60	+0.95	+0.15	+0.75
30°	+0.57	+1.57	+0.05	+1.27
40°	+0.85	+2.45	+0.80	+2.15
50°	+1.87	+3.72	+3.00	+4.02

riments were carried out with a modified Zeiss Parallax-Refractometer. In order to explain the results which appear from Table III the authors have had to divide the refraction of the subjects in two types, A and B. Subjects of type A have mixed astigmatism corresponding to the periphery of the retina and subjects of type B compound hyperopic astigmatism, in both cases with the lowest refractive power along the horizontal meridian. It appears from the table that the refraction in emmetropic eyes and at an eccentricity of 10° deviates at most 0.45 D from the central refraction and on an average considerably less. Not until the eccentricity is 20° will the peripheral ametropia and astigmatism make themselves felt.

According to this the refraction does not

seem to be of any great importance for indirect vision when the eccentricity is less than about 10°.

2. Remarks on the Structure of the retina outside the fovea.

In the remaining part of the retina as well as in the fovea the fineness of the receptor mosaic depends partly on the size and number per unit of area of the receptor cells, partly on the ascending convergence of the fibres (vide pp. 16 and 18).

The receptor cells consist in the layer of cones and rods. In the greater part of the fovea where only cones are to be found the diameter of these increases with the distance from the centre of fovea (conf. table VI p. 109). The centripetally conducting neurones of the fovea have already been mentioned on page 17. Summarily it may be said about these, that there is at least one cone per optic nerve fibre in the fovea, and perhaps more.

Outside a certain boundary in the fovea from an eccentricity of about 26.4 minutes of arc *(Østerberg* 1935 [64]) to 50 minutes of arc *(Koster* 1895) the rods begin to appear, first singly, scattered among the cones, then gradually in greater numbers, while the cones at the same time become fewer. At an eccentricity of 20 to 25 degrees the cones reach their minimum, and at the same time there is a maximum of rods per unit of area, approximately corresponding to the number of cones per unit of area in the foveal centre. Outside this »ring« of rods, the number of rods again decreases.

According to the duplicity theory the cones do not participate in the visual act during dark adaptation and the rods do not participate in case of light-adapted eye. Whether the latter holds good has not been proved with certainty. Experiments made by *Granit* (1941 b) makes it probable that the rods are active both in case of light adaptation and dark adaptation. We shall, however, here assume that the duplicity theory holds.

The afferent neurones from the retina outside the fovea

converge much more than the fibres from the fovea. This appears from countings of the cells in the outer and inner nuclear layer and in the ganglion cell layer at various eccentricities *(Chievitz* 1889). *Polyak* (1936) found likewise by histological investigations on monkeys and apes that the bipolars and the optical ganglion cells in the periphery of the retina are chiefly of the polysynaptic type so that each bipolar is connected with several cones and rods, and again several bipolar cells with one optical ganglion cell. Finally there is a distinct incongruity between the total number of nerve fibres in the optical nerve, about 1 million *(Krause* (1876) according to *Østerberg* (1935 [18])) and the number of cones (about 6.5 millions) and rods (about 118 millions) *(Østerberg* 1935 [88]) the consequence of which is that in the periphery many cones and rods must correspond to one optical nerve fibre, especially if there is one nerve fibre for each cone in the fovea.

3. The Importance of the Psychological Factors, Attention and Practice for Indirect Vision.

Already *Purkinje* (1825 [21, 22]) realized that the psychological factors, attention and practice have a special influence on indirect vision. As soon as a test object appears in the visual field beside the fixation object the subject will have to attempt to divide his attention between the maintenance of an accurate fixation and the perception of the quality of, or variation in, the test object which is to be observed.

Attention may be directed against either large or small areas in the visual field and the visual acuity will vary accordingly. *Purkinje* (1825 [21]) expresses this fact in the following way: »A person is able to concentrate his attention on a single point or line or he may disperse it all over the visual field. These two processes are antagonistic to each other. The more intensely the attention — by central vision — is concentrated on a single point, the more the remaining part of the indirect visual field will disappear from the consciousness; if on the contrary the person is not looking at anything in parti-

cular he will notice more details in the visual field, but perceive the point corresponding to central vision less distinctly.« The same observation is made by *E. Holm* (1923).

How large an area of the visual field, either centrally or peripherally situated, over which the attention may be dispersed without variations in the visual acuity does not seem to have been investigated. Some experiments giving an outline of this problem have therefore been included in this book (vide p. 119).

Besides being dependent on the attention the acuity of indirect vision is also dependent on the t r a i n i n g which the person in question has aquired in observing objects in the visual field with indirect vision. *Dobrowolsky & Gaine* (1876 a) thus found that the best indirect vision was not obtained until after 6 weeks of daily experiments. Furthermore it is probably also due to training that a squinting eye can gradually establish an eccentrically situated »pseudo-fovea« with better visual acuity than other retinal areas of the same eccentricity.

B. Previous Investigations on Indirect Vision.

As described in the introduction it is a common experience that indirect vision in less sharply defined than central vision, although only the outlines of this variation are known. If an attempt is to be made to penetrate further into the physiology of indirect vision, the first task will be to find the boundary between central and indirect vision. The next question to be settled is the rapidity with which the acuity of vision diminishes, and whether all visual functions — in so far as they are comparable — diminishes to the same extent. Not until these problems are fairly settled will it be possible to approach the solution of the central problem, the explanation of the difference between indirect and central vision.

As normal fixation takes place by means of central vision it will be possible, already before dealing with the variation of the visual acuity in the field of vision, to acquire some idea as to the angular extent of central vision, through what is known about the accuracy of the fixation of the eye.

1. The Angular Extent of Central Vision Determined by means of the Size of the Retinal Fixation Area.

When it fixates a point the eye will in spite of the endeavours to fixate carry out some very small involuntary movements. Fixation consists in successive »periods of elementary fixation« each lasting from 1 to 2.5 seconds *(Øhrvall* (1912), *Sundberg* (1918)). During a succession of elementary fixations the image of the fixation point will be carried over various parts of the fovea, larger and more rapid shifts being interposed between the periods. From these shifts the eye does not return exactly to its starting point. During each elementary fixation the eye will also carry out some small quivering, oscillating movements, each being usually less than 50 to 120 seconds of arc and having a frequency of 50 to 100 per second. *(Dohlman* (1925 [113]), *Adler & Fliegelmann* (1934)).

The angular area of the visual field within which the fixation axis thus moves during the fixation of a point may be described as the »error of fixation« or »aberration of the fixation axis« *(Gertz* 1908). The corresponding retinal area is called the fixation area of the retina *(Tschermak* 1931 [1055]).

It is quite evident that if the extent of central vision was smaller than the retinal fixation area the acuity of the visual impression would vary during the fixation. This is not the case. If on the other hand the fixation area was smaller than the area covered by central vision there would be two possibilities. One explanation would be that fixation was attached to certain special receptors within the field of central vision, so that only these receptors were able to give a subjective impression of fixation. If on the other hand such receptors do not exist the movements of the fixation axis within the field of central vision would not be discernible, and the two fields would consequently be identical.

Whether the subjective criterion of fixating a point is the fact that the image of the point falls on specific »fixation receptors« or the fact that the point is more sharply defined in the fixation position than in any other position of the eye cannot be settled on the basis of present knowledge. It cannot therefore be stated decisively whether

the field of central vision on the retina is equal to or larger than the retinal fixation area.

The size of the fixation area may be determined experimentally by objective os well as by subjective methods.

When applying objective methods it is best to record the movements of the eye by means of a narrow beam of light reflected from a small mirror attached to the eye, the mirror carrying the light to a moving photo-film.

By means of such an arrangement *Marx & Trendelenburg* (1911) have found that the movements of the fixation axis during a fixation which lasted for 11.2 seconds did not exceed a visual field with a diameter of 5 min. of arc. *Adler & Fliegelmann* (1934) who used a similar method found a fixation area of the same magnitude, 2.5 to 5 min. of arc.

Another group of authors have found much larger aberrations of the fixation axis. Thus *Øhrvall* (1912) states the diameter to be 23 to 32 min. of arc and *Sundberg* (1918) finds 32 min. of arc. Both series of experiments were carried out by measuring the movement of a scleral vessel, the measurements being taken through an ophthalmometer on a vernier scale. A third author, *Dohlman* (1925 [119]), who has used the above mentioned photographic recording, has found the fixation area to have a diameter of 46 min. of arc.

The reason for this great discrepancy between the results of the experiments may perhaps be found in the fixation objects employed. *Adler & Fliegelmann,* who found the smallest figures, thus used a cross-hair. *Marx & Trendelenburg,* who also found a small fixation field, used a black point on a white surface, but do not state the size of the point. On the other hand the fixation objects used by the authors, who found large values, were all relatively large. Thus the objects used by *Øhrvall, Sundberg* and *Dohlman* were luminous points on dark ground, and the diameter of the points were 3, 4 and 4 min. of arc respectively. The points were approximately twice as large as oscillations of the fixation axis during an elementary fixation, and it would hardly be possible to record a finer fixation adjustment of the eye with such large objects. The size of the fixation objects can, however, hardly explain all

of the difference between the observed fixation fields. It is a more probable explanation that the eyes of the subjects have not been completely light-adapted by the luminous point and that consequently the fixation has not been optimal.

Of all the objective determinations of the retinal fixation area the lowest values (2.5 to 5') seem probably to be most correct.

Subjectively the size of the fixation area may either be determined by simple observation or by experiments. Thus *Javal (Tscherning* 1924 [44]) observed that when two points can be recognized as separated, then either of the points can be fixated. As two points can be seen separately when the distance between them is 2 to 3 min. of arc (vide p. 22) the accuracy of the fixation is probably of this magnitude, as it would otherwise hardly be possible to know which of the points was fixated.

Of experiments only a single series, reported by *H. Gertz* (1908), seems to exist. The experiments are based on the observation that even if a row of points (more than 6) can be recognized as separated, it is not possible to count the points, until the distance between them is somewhat larger. In order to be able to fixate one point at a time, while counting, the fixated point must be seen more distinctly than its neighbours, i. e. only this point comes within the field of central vision. *Gertz* thus assumes that the aberration of the fixation axis and the angular extent of direct vision are equally large.

Working upon this assumption *Gertz* has made some experiments on the basis of which he has calculated that the retinal fixation area and the area of central vision both have an angular diameter between 3'20" and 4'5". This corresponds to those of the objective measurements which have been carried out with the apparently most suitable fixation targets.

If the information as to the extent of central vision on the retina, which the investigations with regard to fixation yields, is summed up, it appears that the diameter of the retinal area of central vision must be either abt. 2.5 to 5 min. of arc or larger.

2. Previous Comparative Investigations on Central and Indirect Vision.

When contradictory results of comparative sense-physiological investigations on central and indirect vision have quite often been obtained, it is reasonable to attempt to explain this by differences in the experimental conditions. Thus the various authors have probably examined different sides of the visual sense, different partial functions which do not vary in the same way with the retinal eccentricity. In the following it is endeavoured to arrange the previous sense-physiological investigations on indirect vision in accordance with the division of the visual sense into partial functions proposed in chapter I. The intention is hereby to give a survey of the problems connected with indirect vision and thus create a basis for new experiments on central and indirect vision.

a. The light sense by central and indirect vision.

As mentioned on page 6 the light sense of the eye is measured by the discrimination factor, i. e. the start intensity divided by the differential threshold. The differential threshold by indirect vision must be assumed to be dependent not only on the light intensity, adaptation level and the size of the stimulated retinal area — as it is the case by central vision — but also on the location of the stimulated area, especially its eccentricity.

The influence of light intensity and adaptation level on the differential threshold, when both central and indirect vision are simultaneously stimulated, has already been dealt with on page 7 Apparently no experiments have been carried out by pure indirect vision and light adaptation.

The dependence of the successive differential threshold on the angular diameter of the test object was investigated with light-adapted eye by *Heinz & Lippay* (1928). Through these experiments the differential threshold was shown to be dependent on the area to a greater extent than by central vision.

The first measurements of the variation of the differential

threshold with the retinal eccentricity were carried out by *A. Hueck* (1840). *Hueck* found the value of the differential threshold by determining the size which a black spot must have in order to be just visible on white ground. In this way it could be shown that the threshold was higher for an eccentricity of 1° than it was centrally, and that it increased uniformly with increasing eccentricity. This result has later on been confirmed by *Groenouw* (1893) and also by the investigations on which the clinical perimetry is based (conf. e. g. *Bjerrum* (1889), *Ronne* (1915)). In all these experiments the difference in brightness between object and background has been kept constant and the angular size of the object varied. As the differential threshold decreases with increasing size of object, it will be found — when using this method — that the differential threshold does not increase with the eccentricity to such an extent as if the size of the object was kept constant.

Of experiments with varied difference in brightness between object and ground only a few have been carried out with perfectly light-adapted eye. For this reason a paper by *Guillery* (1897 a) must thus be rejected. *Dobrowolsky & Gaine* (1876 b) have found, through perimetrical experiments with a white *Masson's* disc with a ringshaped shadow of variable intensity, that the differential threshold increases with the eccentricity already within the macula lutea. Against this statement the following objections may, however, be made. Firstly, it is doubtful whether such a large object as a *Masson's* disc should be used in the distance chosen at such small displacements in eccentricity. Secondly, no readings closer to the centre than 5° are given in the said paper. At 5° the differential threshold is almost twice as large as centrally. With a similar method *Bjerrum* (1882 [105]) found the same result, while *Exner* (1870) found the same threshold for central vision as for an eccentricity of 5°. A direct explanation of this cannot be given. *Dobrowolsky & Gaine* themselves state the reason to be that *Exner's* experiments were carried out by artifical light (gas light), but this is, however, hardly sufficient explanation.

No measurements of the intensity threshold with light-adapted eye seem to have been carried out.

b. The colour sense by central and indirect vision.

As colour sense is defined as the ability to recognize and discriminate colours, comparative experiments as to colour sense by central and indirect vision might consist in determinations of the threshold for recognizable difference in wave length with varying eccentricity. Such experiments have apparently not yet been carried out with pure spectral colours and light-adapted eye.

In addition to this it would also be of interest to examine a series of partial functions, as f. inst. the differential threshold and the min. separabile for light of various wave lengths. Before carrying out such experiments it will be natural to account for the processes of the various functions with achromatic light. For practical reasons the present book does not go beyond this, and it does not intend to deal with further problems of colour sense.

c. The physiological resolving power by central and indirect vision.

The min. separabile. Most comparative investigations on the min. separabile by central and indirect vision have been carried out with objects illuminated from before and of various kinds, white or black dots, squares or lines on black or white ground respectively. As to the angular extent of central vision these experiments yield only uncertain information. Only *Burchardt* (1882) has taken readings sufficiently close to the centre, and found that the min. separabile was the same in the centre as at a distance of 15 to 20 min. of arc from the centre, but already at an eccentricity of 30 min. of arc the min. separabile was twice as large. Against *Burchardt*'s experiment an objection may, however, be put forward, namely that the object was not two points but a group of points (4 to 6) and thus relatively large in comparison with the eccentricities used (*Guillery*, 1897 b). At larger eccentricities

the min. separabile increases rapidly, although the statements as to the rapidity given by different authors vary considerably (conf. *Hueck* (1840), *Volkmann* (1846 [334]), *Aubert & Foerster* (1857), *Königshöfer* (1876 [12]), *Groenouw* (1893), *Guillery* (1897 b)). *Aubert & Foerster* (1857) as well as *Groenouw* state that the rate of increase is larger in the vertical meridian than in the horizontal.

At *Aubert & Foerster*'s experiments the peculiar phenomenon established itself that the min. separabile by constant angular size of object was smaller for indirectly seen objects, if they were closer to the eye, than if they were more distant. (The so-called *Aubert-Foerster phenomenon*). According to recent investigations (e. g. *Musyleu* 1937) the explanation of the phenomenon seems to be that Aubert & Foerster did not keep the size of the background of the object constant. (conf. p. 36).

The min. separabile for twin objects illuminated from before can, however, according to the preceding chapter (vide p. 20) only be considered a function of the differential threshold of the intermediate area. Only through experiments with luminous points of threshold brigtness on unilluminated ground a measurement of the size of the functional units will be obtained. It would of course be of great importance for our knowledge about indirect vision to follow a possible variation of the diameter of the functional units in the visual field from the fixation point and outwards. However, the literature offers only few investigations on the min. separabile for luminous objects, and at none of these papers do the experimental conditions fulfill all the requirements raised.

Only a single series of experiments made by *Wertheim* (1887) was carried out with luminous points as test objects. The points consisted in a system of pin holes in a tin foil illuminated from behind. On the basis of *Wertheim*'s tables the values of the min. separabile which appear from fig. 2 a can be calculated. It is seen that even at an eccentricity of 30 min. of arc the min. separabile is larger than it is centrally. As the experiments were not carried out in complete light adaptation, and as the brightness of the points has probably exceeded threshold brightness considerably, the result must be taken with some reservation. The same applies to some other experi-

ments by the same author (1894) with a grating illuminated from behind as object. Through these experiments *Wertheim* found that the min. separabile increased gradually when the eccentricity was increased from 2.5° to 70°. The increase was

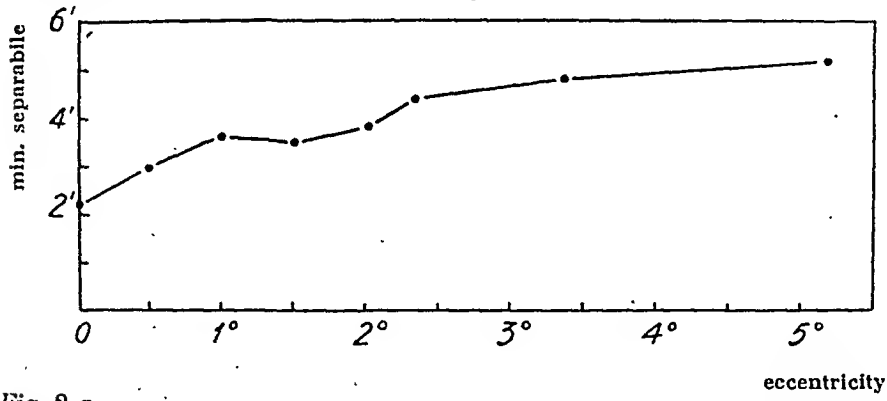


Fig. 2 a.

The min. separabile for luminous points at varying retinal eccentricity, according to *Wertheim* (1887).
Abscissa: Eccentricity retino-temporaliy in degrees.
Ordinate: The min. separabile in minutes of arc.

smallest in the horizontal meridian, especially in the retino-nasal, and largest in the vertical meridian, especially in the retino-inferior. Later on *Weymouth* et al. (1923 and 1928) has reported similar experiments with light-adapted eye (vide

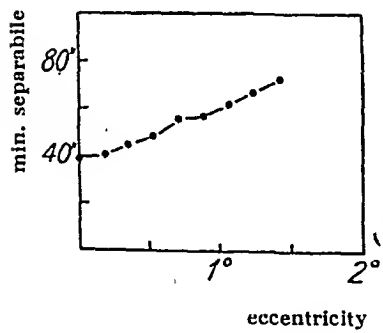


Fig. 2 b.

The min. separabile for a luminous grating at varying retinal eccentricity, according to *Weymouth et al.* (1928).
Abscissa: Eccentricity in degrees.
Ordinate: Mean value of the min. separabile in the four principal meridians in seconds of arc.

fig. 2 b). *Weymouth* is the author who has measured the min. separabile nearest to the fixation point (at 21.7 min. of arc and with one subject at an eccentricity of 10.6 min of arc), but the readings at 10.6 min. of arc must be considered to be rather doubtful, as the object was rather large (diameter 10 min. of arc). The light intensity used seems, however,

also in *Weymouth*'s experiments to have been considerably above threshold brightness, just as the dark ground between the bars of the grating — according to the description of the method — has hardly been absolutely unilluminated.

As thus none of the investigations in question have been carried out under such well-defined conditions that the min. separabile measured can be considered to be a measure for the functional units, new experiments will be desirable.

Perception of movement and form sense. As mentioned in chapter I both perception of movement and form sense can — in addition to the min. separabile — be taken as a measure for the physiological resolving power.

Comparative experiments with regard to perception of movement by central and indirect vision was published by *Basler* (1908), who determined the minimum recognizable movement of a luminous slit. The threshold was of the same size in the centre of the visual field and up to an eccentricity of 3° when it began increasing. As the experiments were not carried out in light adaptation and not with an object consisting of a luminous point on unilluminated ground but of a luminous slit in a visual field either filled with illuminated objects or completely dark, the result cannot be taken to be a function of the physiological resolving power.

The third partial function the relation of which to the retinal structure seems rather simple, the form sense, has never been investigated by indirect vision. It will therefore be of interest to examine both form sense and perception of movement at varying eccentricity in the visual field, at the same time verifying whether the relations of these functions to the min. separabile conform to the theories propounded in chapter I (vide chapter V and VI for further details).

d. Variation of the complex functions by central and indirect vision.

Aligning power. By all investigations on the aligning power at various eccentricities a gradual increase in the displacement threshold has been observed within the area examined from the fixation point to an eccentricity of 20°,

Table IV.

The displacement threshold at varying retinal eccentricities.

Author:	*Bourdon* (1903) (quoted by *Hofmann* (1925, [57]))	*Hofmann* (1925, [57])
Test object:	Two black lines on white ground which can be displaced in relation to each other.	A square surface, one half white, the other black. The two halves of the dividing contour can be displaced in relation to each other.
Eccentricity:		
0	7"	9"
1°	23"	
2°		54"
5°	3'57"	2'3"
10°	6'53"	3'48"
20°	13'56"	

(vide table IV). The authors have apparently not attempted to determine the angular extent of central vision through these experiments. For this reason there are no readings at eccentricities smaller than 1°, apart from the measurement carried

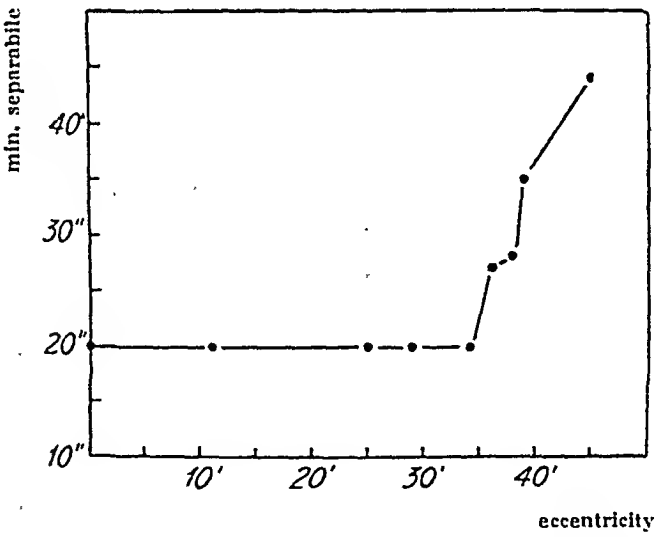


Fig. 3.

Discrimination of thickness of line at different retinal eccentricities. According to experiments made by *Adler & Meyer* (1935).
Object: A dark slit in a white ground. The total length of the slit 23'20".
Abscissa: Eccentricity, retino-nasally, in minutes of arc.
Ordinate: The increase in width of the variable half of the slit in seconds of arc.

out by central vision. New experiments would therefore be desirable, (vide chapter VII).

Discrimination of thickness of line. The only investignations in the literature at hand on the variation of this function with the retinal eccentricity are made by *Adler & Meyer* (1935) and are of special interest, as the experimenters succeeded in proving the existence of a »physiological fovea« of the same size as the anatomical, vide fig. 3 and table XV page 169. The object employed was a dark slit in a white ground illuminated from before. The slit (which had a total length of 23'30") was divided in two equally large parts, in such a way that the width of each half could be varied independently of the other. A black dot on white ground was used as fixation target. By means of an electric switch, operated by a motor, the fixation target and test object was illuminated alternately, 2 and 0.15 seconds respectively. The adaptation level was kept constant during the experiment, but it was only dependent on the relatively small background for the objects. (The diameter is not stated, but according to the illustration it seems to have been abt. 1.5°.) Whether the eye of the subject has been fully light-adapted cannot be decided.

The experiments were carried through with one subject in the four principal meridians, and for each of these the threshold for recognizable increase in thickness was found to be the same within a radius of 42 min. of arc from the centre (0.125 mm on the retina). If this observation of a »physiological fovea« could be verified, it would mean that the simultaneous intensity discrimination was the same everywhere in the fovea, and it would be the first time that it had been possible to prove the existence of a physiological fovea in a light-adapted eye. In chapter VIII it has therefore been endeavoured to repeat *Adler & Meyer's* experiments and further to investigate the perception of thickness of line under various experimental conditions.

The minimum legibile. In spite of the complex nature of the min. legibile it is often used for investigations on the physiological basis of indirect vision, e. g. by *Aubert &*

Foerster (1857), *Dor* (1873), *Königshöfer* (1876[25]), *Schadow* (1879), *Bloom & Garten* (1898) and *Ruppert* (1908). By all these experiments a decrease in visual acuity with increasing retinal eccentricity has been observed, the rate of decrease differing somewhat according to the various series of experiments. The only author who has measured the min. legibile at an eccentricity as small as 1° is *Königshöfer,* who found the visual acuity here to be $^1/_4$ to $^1/_3$ of the central visual acuity.

C. Theoretical Explanation of the Difference between the Characteristic Features of Central and Indirect Vision.

When endeavouring to find the reason for the difference between central and indirect vision, it would be natural to look for it among the optical, structural and psychological principles of indirect vision.

Among the optical factors importance has been attributed in the first place to spherical aberration *(Young* (1801[144])), secondly to reduced illumination of images on the peripheral parts of the retina *(Landolt & Nuel* (1873)), and finally to difference in refraction. Apart from the fact that imperfections in the optical imagery no doubt have some influence at the periphery of the retina, they cannot — as assumed by *Volkmann* (1846[334]) and *Fazakas* (1928) — be considered to be the main reason of the difference between central and indirect vision. This appears especially from the fact that no essential difference in refraction or astigmatism can be found at eccentricities within abt. 10° (vide p. 52), while the visual acuity decreases rapidly at any rate from the border of the fovea and outwards. Moreover it will hardly be possible to explain the different variation of the visual acuity along the four principal meridians by optical factors.

Ever since *E. H. Weber* (1846[526]) correlated the resolving of two points to the structure of the retina by propounding the term »Empfindungskreis« (vide p. 19), attempts have been

made to explain the lower visual acuity of indirect vision by the structure. But while *Weber* himself ascribed most importance to the distribution of the nerve fibres, *Aubert & Foerster* (1857) assumed that the number of cones per unit of area in the various parts of the retina could provide the explanation. *Aubert & Foerster* regretted that no accurate countings, the results of which could be compared with the min. separabile, existed at the time when their experiments were carried out.

Later on *Wertheim* (1887) compared the min. separabile for luminous points with countings of the cones carried out by *Salzer* and found conformity between the min. separabile and the number of cones up to, but not outside the border of the fovea. In addition to the objections against *Wertheim*'s method, mentioned on page 62, it must be stated that when his calculations are examined it is found that the value of the central min. separabile of 2.2 min. of arc will correspond to abt. 11 μ. As the diameter of the cones are three to five times smaller, the conclusion drawn from *Wertheim*'s experiments must be that in the foveal centre the functional units consist of a certain number of cones (3 to 5), while in the remaining part of the fovea they consist of the same number of cones of larger size, however.

It is known from histological investigations (vide p. 53) that the convergence of the afferent pathways is much more pronounced in the periphery of the retina than in the fovea, but the eccentricity at which this increase of convergence begins is not known, and it can only be determined by comparing the size of the functional units (determined through the min. separabile for luminous points of threshold brightness on unilluminated ground) with histological countings of the frequency of cones per unit of area.

It cannot be taken for granted that there are functionally separated units at all in the peripheral parts of the retina. The problem may be solved in two ways. In the first place it might be determined whether the min. separabile for minute luminous squares of threshold brightness on unil-

luminated ground is independent or not of the size of the squares by indirect vision, as it is by central vision (conf. p. 23). If not independent, it would suggest that anatomically separate, independent functional units do not exist. Descriptions of experiments on this question will be given in chapter IV page 132. Secondly the min. separabile might be compared with the perception of movement. From the results of previous experiments it is generally assumed that the distribution of cones and nerve fibres cannot explain why the perception of movement seems so extremely distinct compared with the spatial perception, otherwise so obtuse and indistinct by indirect vision *(v. Fleischl* (1883), *Ruppert* (1908), *v. Helmholtz* (1896[264]), *Hofmann* (1925[70])).

It is, however, doubtful whether it is possible to draw any conclusions from the comparative experiments on the min. separabile and similar functions on one hand and perception of movement on the other (e.g. *Exner* (1875), *Aubert* (1883) and *Ruppert* (1908)). The authors mentioned determined the minimum angular velocity, which is perceptible as movement, and compared the variation of this threshold with the visual acuity in the same meridian. Such a comparison between a velocity threshold and the min. separabile is hardly permissible when the test objects differ considerably in size, as it is the case in these investigations. The smallest distance which a luminous point must travel in order to render the movement recognizable is the only measurement which is comparable with the min. separabile, and which can be related to the retinal structure (conf. p. 38). Such experiments have been carried out and recorded in chapter V.

Two theories have been advanced in order to explain, why the perception of movement by indirect vision is relatively better than the visual acuity. One explanation has been given by *v. Fleischl* (1883). He assumed that the cones in the peripheral parts of the retina were coupled in groups, the size of which increased with increasing eccentricity, and each of which was connected with one nerve fibre. The cones of each group did not form anatomi-

cally separate units, but was to some extent mixed with cones belonging to adjoining groups. This would have the effect that the visual acuity became rather poor, while even a small displacement of a retinal image would be perceptible, as soon as the image moved from a cone of one group to one of another. It must, however, be noticed that, if this held good, a moving point would — to the observer — appear to jump forwards and backwards, and this is contrary to experience.

In his discussion of this theory *Hofmann* (1925 [70]) overcomes this objection by asuming that the optical imagery at the periphery is so imperfect that even a point has a retinal image, which stimulates several cones at the same time. It is moreover assumed, that the greater the number of stimulated visual cells in a group, the higher its excitation level, and that the state of excitation of two neighbouring groups has a certain influence on the subjective localization of the object. In that case it would be possible to recognize a difference in position within the area in which the cones from two groups are mixed, according to whether a larger or smaller number of cones belonging to either of the groups are stimulated. The effects of practice on indirect vision is likewise explained by the assumption that the eye is gradually trained to recognize steadily diminishing differences in the stimulation of two neighbouring groups *(Hofmann,* 1925 [70]).

The other theory was propounded by *v. Helmholtz* (1896 [264].) »Imagine a surface covered by receptor cells the corresponding nerves of which are dissolved into a fine plexus of anastomotic nerve fibres, which on one side is connected with the numerous receptor cells and on the other with the cerebral cortex by means of a much smaller number of nerve fibres. It is further assumed that the stimulation of a receptor cell is transmitted through the plexus to the roots of the nearest nerve fibres, but in decreasing degree according to this distance.*) Under these conditions any point of the plexus

*) Confer *Hartline*'s (1940) experiments by which it was shown that in the retina of amphibia the receptive field corresponding to one optic nerve fibre is most light sensitive in the centre, i. e. the impulses here are of the highest frequency.

would be responsive, and the sensation caused by the stimulation of a point which falls within the starting points of these nerve fibres would differ according to the position of the point in relation to the three starting points.. If the eye was able to recognize minute differences in the stimulation of such nerve fibres, it would also be able to recognize minute displacements. But the stimulation of two points situated in the same area would only give the impression of one point.«

If a closer comparison of the theories of *v. Fleischl — Hofmann* and *Helmholtz* is carried out, it will be seen that they only differ as regards anatomical details. Both assume that the stimulation of a single receptor cell *(Helmholtz)* or a few receptor cells *(Hofmann)* will cause a relatively high excitation level of one optical ganglion cell and a lower one of the adjoining cells. While *Helmholtz* in order to explain this assumes the existence of an anastomotic plexus of nerve fibres, something like the synapse between the bipolars and the optical ganglion cells, *v. Fleischl* and *Hofmann* hypothesizes that the receptors corresponding to one ganglion cell are mixed with the receptors of the adjoining ganglion cells in a common boundary zone. In principle *Helmholtz's* theory seems to be the most acceptable. The existence of an »anastomotic plexus« of nerve fibres has, however, never been proved, but it is probable that an impulse may be transmitted from one ganglion cell to the adjoining ones through the association elements of the retina. These include both amacrine cells and horizontal cells, most numerous in the fovea however, as well as mutual overlappings of the dendrites of bipolars and optical ganglion cells. This overlapping is found not only in the periphery but also in the fovea *(Polyak,* 1936).

According to this the anatomical structure of the fovea should also be so as to allow a dispersion of the impulse, which might seem strange considering the high visual acuity of the fovea. This problem, as well as the above described theory by *Helmholtz,* has been elucidated, and the theory has been verified in principle by the electro-physiological investigations of the latest decennia.

Electro-physiological experiments on central and indirect vision.

Before correlating the results of the electro-physiological investigations of recent years to the theories about indirect vision, it will be appropriate to give an outline of the chief features of the electro-physiology of the optic nerve and the retina. The representation is based on a number of recent experiments with recording of electrical discharges from the optic nerve as a whole or from single nerve fibres, while experiments in which the electrodes are placed one on the cornea and the other on the fundus or the optic nerve, so-called »electro-retinography« has not been taken into consideration.

By placing electrodes at two points of the optic nerve or a bundle of fibres it will be possible, by the use of sufficient amplification, to record a rythmical electrical discharge passing down the nerve, the frequency of which depends on the retinal illumination. Such action currents were first observed by *Adrian & Matthews* (1927 a) in the eyes of eels and frogs and by *Granit* (1933) in the eyes of mammals.

Under the influence of a constant stimulus*) (e. g. light) on the eye a rapid succession of action currents of approximately uniform size appears, if the intensity threshold is exceeded. This stream of impulses starts after a certain latent period. At first the train of action currents shows a vigorous outburst of activity (»on«-effect), but even if the stimulus remains constant, this is followed by a rapid decline in frequency. When the stimulus is removed, there is a renewed outburst of impulses (»off«-effect). After the first outburst action currents of low frequency will continue as long as the stimulus lasts — the frequency sometimes decreasing an account of adaptation to stimulus. While the frequency rises considerably with increased illumination or increased stimulated area, the individual changes of potential are of the same magnitude.

*) »We will define a stimulus as any change in the environment of an excitable tissue which, if sufficiently intense, will excite the tissue i. e., will cause it to display its characteristic activity«. (*Adrian* 1928 [18]).

The experimental results hitherto mentioned has been obtained by recording the discharge from the whole of the optic nerve or larger parts of it. The response of single nerve fibres has, however, given more differentiated information about the optic nerve currents. Thus *Hartline* (1938) found three sorts of optic nerve fibres in the eyes of amphibia, fishes and reptiles, each with its type of discharge. The first type, which constitutes 20 per cent of the optic nerve, reacts — on the retina being illuminated — with an initial outburst followed by action currents of low, constant frequency. The second type, which amounts to 50 per cent, gives both an »on« and an »off« effect, but no impulses in the meantime. Finally from the remaining fibres (abt. 30 per cent) an »off«-effect only is observed, the train of impulses dying off gradually during several seconds. If the retina is reilluminated during the after-response, the latter is immediately discontinued.

Through the experiments previusly mentioned *Adrian & Matthews* (1927 a) found — besides the facts already dealt with — that if the light source — and consequently the image on the retina — was moving about, it would act as a more intense stimulus than if it was stationary. This must probably be explained in the same way as the fact that a momentary exposure gives a higher frequency of impulse than continuous illumination *(Adrian & Matthews* 1927 a), and that the brightness of sensation is likewise highest for momentary illumination *(Broca & Sulzer* (1902), *Lehmann* (1905[183])).

Adrian & Matthews (1927 a) assume that the fact, that an increase of the area stimulated has the same effect as an increase of the light intensity, is due to a dispersion of the state of excitation in the retina, whereby several ganglion cells are enabled to give a response.

In a more recent paper *Adrian & Matthews* (1928) succeeded in proving an interconnection, an »interaction«, between stimulated retinal areas. If, at the same thime, four separate retinal areas were illuminated the latent period would become shorter, than if each of the four areas had been illuminated independently. It will hardly be possible to explain this in any other way than by an interaction through the

transversal pathways in the retina with the described effect. The latent period depends on the excitation level. Thus an increase in frequency conditioned by an increase in area or intensity is always accompanied by an abbreviation of the latent period *(Adrian & Matthews* 1927 b). In confirmation of the theory about stimulation through the transversal pathways *Graham* (1932) found that in the eye of the limulus, which consists of omnatidia without transversal connections, the latent period is independent of the number of omnatidia stimulated. But it is perceptibly abbreviated when the light intensity is increased.

For the human eye it would also be natural to explain the dependence of the differential threshold on the light intensity and the area stimulated (vide pp. 7 and 8) as a result of a similar interaction as the one observed by the »four-point experiment« *(Adrian & Matthews* 1927 a). As the dependence on the area is more marked in the retinal periphery than centrally (vide p. 59), it seems to indicate that the interconnection between the ganglion cells is more pronounced in the periphery.

Investigations on the fusion frequency of flicker in humans also suggest the existence of an interconnection between separated areas, and that this interaction is different by central and indirect vision. When a fusion of intermittent flashes of light occurs, it probably means that the discharge in the optic nerve is of constant frequency, as it is in case of continuous illumination, just as alterations in the fusion frequency probably originates in the retina *(Granit* 1936[54]). When the stimulated area or the light intensity is increased, the fusion frequency is decreased and more so at the periphery than centrally *(Granit & Harper* 1930). It appears from the same paper that on measuring the fusion frequency for two semi-circular objects separated by a variable intermediate space it was found to increase when the objects were approached to each other. The variation is most marked at the periphery, but exists at the centre as well. When the visual acuity of the fovea is good in spite of this interaction, it is, according to the authors, due to the fact that here the inter-

action has an effect on the stimulated areas only, not on the intermediate, non-stimulated area. This is assumed to be due to certain inhibitory factors only to be found corresponding to the fovea.

In a somewhat later paper *Graham & Granit* (1931) has found further confirmation of this through experiments with a test object similar to the one applied by *Granit & Harper* (1930). The brighness of each of the two semicircles could be varied separately. The frequency of flicker of each object could also be varied separately or could be synchronized with each other. (The objects had a radius of 30 min. of arc, and the distance between them was 4 min. of arc. The intermediate space served as fixation target). When the two half parts had the same brightness, and the flicker synchronized, they gave a higher fusion frequency than if each of them was taken separately. If only one object flickered, and the other was of the same brightness, this would give an increase in the fusion frequency of the first object, but a smaller increase than if both objects flickered. It has thus been shown that a continuously illuminated object can increase the fusion frequency of another object clearly separated from the first, and this must be due to an interconnection between the illuminated areas. If during the experiments the brightness of one object was diminished the fusion frequency of the darker object either remained constant or was diminished by the presence of the brighter one. The fusion frequency of the brighter semi-circle was vice versa increased by the presence of the darker object, if the difference in brightness was not too large. The fact that the brighter object does not seem to influence the dark one was explained by *Graham & Granit* by the inhibitory influence (inhibition) from the highly illuminated retinal area. Such inhibition could not be traced by corresponding experiments in the periphery (at an eccentricity of 10°).

On a basis of these experiments *Graham & Granit* assume that the existence in the periphery of an interconnection in the above mentioned sense can explain the reduction of the visual acuity and intensity discrimination at such eccentricities where

the convergence of the fibres of the visual pathway and the defective imagery does not offer sufficient explanation.

While discussing the theory by which *Helmholtz* explained the features of indirect vision (vide p. 71), it was pointed out that the main idea of the theory is the assumption of a dispersion of the impulses in the retinal periphery. Such dispersion has been recorded by the electro-physiogical experiments and the investigations on the fusion frequency of flicker mentioned above, so that these experiments confirm *Helmholtz'* theory.

PART II

AUTHOR'S INVESTIGATIONS, COMPARATIVE SENSE-PHYSIOLOGICAL EXPERIMENTS ON CENTRAL AND INDIRECT VISION

Survey of Part II.

It appears from the outline of previous experiments on indirect vision given in the preceding chapter, that the methods of none of the experimenters have been such as to satisfy the requirements raised. The test object, and consequently the visual function examined, often seems to have been chosen somewhat casually. Usually the eyes of the subjects have not been perfectly light-adapted, or the test objects have been so large that they were not suitable for fine measurements, e. g. of possible variations within the fovea. Finally — and most essentially — none of the experimental methods have been such as to condition a determination of the size of the functional units.

It is consequently no wonder that the experiments on indirect vision are rather incomplete, not the less because the knowledge of the functional units by central vision has been very slight up to the last years.

It was previously a rather wide-spread conception among sense-physiologists that the minimum separabile is always a function of the intensity discrimination of the retina and not of the fineness of the retinal mosaic (e. g. *Bjerrum* (1882 [3]), *Hofmann* (1925 [28]), *Guillery* (1931 [763])). The fact, that the minimum separabile for luminous points of threshold brightness on unilluminated ground is a measure of the diameter of the functional units, was only proved by *Berger & Buchthal*'s experiments (1938 a). After this it was natural to examine indirect vision with the same technique. Such experiments were carried out and the results recorded in chapter IV. These experiments offered an opportunity both of verifying the principal results

obtained by *Berger & Buchthal* — which apparently has not been done before — and of further substantiating the results on certain points.

The object of the experiments on the minimum separabile was to examine this function under different experimental conditions in order to collect sense-physiological measurements which, when compared with our knowledge of the retinal structure, could yield some information as to the function of the retina. Luminous points on unilluminated ground should be especially suitable test objects, as the min. separabile in this case — at any rate by central vision — should be a measure of the average diameter of the functional units.

In the following the main points of the investigation shall be shortly summarized.

A. The minimum separabile for luminous squares is measured at various retinal eccentricities. The size of the squares chosen for the experiments is so small that the squares are seen as points. The aim of the experiments is to solve the following questions:

1. Hov large is the central min. separabile for luminous points of threshold brightness on unilluminated ground? (conf. p. 23).
2. What is the extent of central vision on the retina (conf. pp. 55 and 58).
3. Does the min. separabile vary uniformly whith the retinal eccentricity, or is it dependent on structural limits, especially the extent of the rod-free area?
4. How great is the accuracy with which the min. separabile may be determined by central and by indirect vision?

B. Comparative measurements of the min. separabile in various retinal meridians are carried out, the minimum eccentricity at which a difference may be verified being f. inst. determined.

C. The influence of attention on the min. separabile is examined, both by central and by indirect vision. The aim of the experiment is to determine the extent of the central area in the visual field to which the attention may be applied, without the central visual acuity deteriorating (conf. p. 55).

D. The dependence of the min. separabile on the retinal illumination is examined by central and indirect vision by means of experiments on the influence of intensity and exposure time.

1. The importance of intensity. Does the min. separabile for luminous squares on unilluminated ground increase with the retinal illumination as found by *Berger & Buchthal* (1938 a), and if so, does this apply to indirect as well as central vision?

   Is the dependence another for very large squares, where the min. separabile is assumed to be a function of the intensity discrimination?

2. Continuous illumination and momentary illumination. Does the duration of the exposure influence the min. separabile for small luminous squares on unilluminated ground, and is the dependence the same by central and by indirect vision?

E. The dependence of the minimum separabile on the size of square. The experiments are carried out with luminous squares of threshold brightness on unilluminated ground, with unilluminated squares on luminous ground and with test objects illuminated from before.

The results obtained by *Berger* (1936 and 1939) and by *Berger & Buchthal* (1938 a) for central vision are verified, and similar experiments at various eccentricities are also performed. If the dependence varies with the eccentricity it is presumably possible to draw conclusions from this fact about the maximum distance from the centre at which separated functional units can be found (conf. p. 68).

The very few previous comparative investigations on central

and indirect vision have usually dealt with a single visual function only. In the present investigation, however, the visual capacity of the subjects have been tested by means of a series of different functions under experimental conditions which were as far as possible kept constant. By this procedure it has been possible to investigate various aspects of the visual sense, and to do it in such a way that the experimental results are numerically comparable to a much higher degree, than it is usually the case.

In addition to the experiments on the min. separabile investigations were also carried out on the perception of movement and the form sense, which can both, according to chapter I, be considered to be a measure of the physiological resolving power under certain conditions.

By the experiments on perception of movement it was attempted to solve two questions:

A. Firstly, it is attempted to verify that the perception of movement, as maintained in chapter I, is a measure of the physiological resolving power. The distance which a diffraction disc (p. 15), having an oscillating movement on the retina, must travel in order to render the movement recognizable is determined by central vision. The result is employed in verifying the theoretical calculation carried out on page 38 of the same figure on a basis of the diameter of the retinal image and the excursion of the oscillating movements of the eye.

B. Secondly, the variation of the perception of movement with the retinal eccentricity is examined. If, by indirect vision, the perception of movement is relatively »better« than the physiological resolving power measured by the min. separabile, then it indicates a dispersion of the impulse (vide p. 71).

As to the results refer to chapter V.

The third function which, according to chapter I (page 41), can be used as a measure of the physiological resolving power — although with a certain reservation — is the form sense. A summary of the experiments on this function is given below:

A. The form sense is measured at various retinal eccentricities. The visual angle which must be subtended by an equilateral triangle or a square in order to make it possible to recognize the angularity of the figure is compared with the min. separabile for luminous points on unilluminated ground.

1. If the side of the polygon is found to be more than twice the min. separabile, it will serve as a confirmation of the theory advanced by *Berger & Buchthal* (1938 b) about the alternating activity of the functional units.
2. If the form sense is essentially a measure of the physiological resolving power, it should be dependent on the retinal eccentricity in the same way as the min. separabile.
3. The accuracy with which the form sense may be measured by central and indirect vision is determined.

B. The dependence of the form sense on the retinal illumination is examined.

1. By experiments centrally in the visual field the dependence is examined within an intensity range wider than the one employed by *Berger & Buchthal* (1938 b), who has made experiments with threshold brightness and a retinal illumination of 2 lux. The aim was to collect more detailed information as to the influence of the illumination.
2. Similar experiments are carried out at an eccentricity of 5°, in order to find out whether the dependence is the same as in the centre.

The results of the experiments are given in chapter VI.

In addition to the experiments on the physiological resolving power the complex functions, aligning power and discrimination of thickness of line, were investigated in the same way by central and indirect vision.

The experiments on aligning power, which is probably a function partly of the intensity discrimination and

partly of the physiological resolving power (vide p. 43) had two aims.

A. As the dependence of the aligning power on the retinal eccentricity has not previously been determined at small eccentricities, and as it is impossible to foretell the results, experiments were performed for this purpose, while the accuracy with which the displacement threshold can be determined was found through corresponding experiments.

B. When determining the displacement threshold for two slits of steadily decreasing length, a time will be reached, when the slits have become so short that it is no longer possible to recognize their oblong shape, and the function investigated will now be the min. separabile for two points. It is certain that under these conditions the displacement threshold will increase, but it is not possible to predict the slope of the curve. If the test object consists of two luminous slits of threshold brightness in an unilluminated ground, the dependence on the length of the slits will be of special interest, as the experiment in that case will show the transition from a complex function (the aligning power) to a simple function (the min. separabile for luminous points). Experiments of this kind were consequently carried out by central and indirect vision.

As to the results reference should be made to chapter VII.

The last mentioned of the functions examined, the discrimination of thickness of line, is measured by the smallest perceptible alteration of the thickness of one out of two lines or slits in alignment with each other. According to the theories advanced in chapter I (p. 44) the discrimination of thickness of line is a function of the intensity discrimination of the eye, when the two lines are above a certain length. Through the experiments the influence of three factors on the discrimination of thickness of line was investigated.

A. *Adler & Meyer* (1935) have examined the dependence on the retinal eccentricity and have found that the discrimination of thickness of line is the same within

a »physiological fovea« with an outer limit at an eccentricity of 42' (vide p. 66). As it has been impossible to observe a corresponding dependence for the other functions investigated, an attempt was made to reproduce the experiment made by *Adler & Meyer,* under the same experimental conditions as well as under modified conditions.

Similar experiments were carried out in order to find the accuracy with which the discrimination of thickness of line may be determined.

B. The influence of the retinal illumination is investigated in order to be able to compare the result with the dependence of the intensity discrimination on the intensity.

C. Finally the importance of the dimensions of the lines (i. e. length and thickness) for the values found is determined.

The experimental results are recorded in chapter VIII.

In a supplementary chapter IX an account is given of some experiments on *Troxler*'s phenomenon by central vision in order to elucidate the theory mentioned on page 179 about an alternating activity of the retinal functional units.

In the following chapter an account is given of the experimental methods employed, general experimental conditions being dealt with first and secondly the special arrangements by means of which the individual visual functions were examined. Finally the course of the experiments and the calculation of the results is described.

Chapter III

EXPERIMENTAL METHODS

A. General Experimental Conditions.

Subjects. All experiments were carried out monocularly with three subjects, in some cases with only two subjects, however. All experiments in which the absolute value of the thresholds read was considered important were carried out with subjects thoroughly trained in reading by indirect vision.

The visual acuity and refraction of the subjects, determined by means of *Snellen*'s test chart, appear from the below table:

Subject:	*Visual Acuity and Refraction:*
S	6/6 Emmetropia
G	6/6 Emmetropia (—0.50 sph. see below)
M	6/6 +0.50 cyl.ax. 80°
V	6/4.5 —0.50 cyl.ax. 0°
A	6/4.5 Emmetropia

In the subject G a myopia of 0.50 on the eye employed (the right eye) was found by a renewed examination after the course of two or three years, while the other eye remained emmetropic. This slight myopia did not seem to trouble the subject, when using an »artificial pupil« (vide following page) during the experiments. It was therefore judged to be permissible to use all the experiments made with this subject, especially as these experiments did not in any way deviate from corresponding experiments with other subjects. The subject was not, however, used after the myopia had been ascertained. The slight astigmatism observed in the subjects M and V was also judged to be negligible, although they never wore corrective glasses.

Light adaptation. In these experiments, just as in all of *Berger*'s and *Berger*'s *& Buchthal*'s experiments, much stress was laid on the condition that the eyes of the subjects were constantly and perfectly light-adapted. Light adaptation was ensured by placing a screen painted a dead matt white, illuminated by 14 25-watt lamps, in front of the subject. In the screen a square hole was cut out through which the fixation target and test object could be seen. The white screen illuminated the pheripheral part of the visual field from an eccentricity of 30—40° and outwards. Further two 75-watt lamps were suspended behind the head of the subject, assisting in maintaining light adaptation a. o. by illuminating the metal disc with the artificial pupil from behind (wide below). The illumination before the eye was measured by a photocell luxmeter to be 140 lux. All lamps were screened in the direction of the test object, so that only the small amount of light reflected from the face of the subject could reach the distant test object.

Artificial pupil. In order to eliminate the effect of any variation in pupil area all experiments were carried out with an artificial pupil placed in a trial frame abt. 12 mm in front of the eye examined. The artificial pupil consisted of a matt black circular metal disc with a central hole having a diameter of 3 mm. By means of this size of pupil almost maximum optical resolving power was obtained (vide p. 15). By means of screws on the trial frame the artificial pupil could be placed in any desired position before the eye. The subjects were taught to centre the artificial pupil in relation to the visual axis. A black metal disc without hole was placed in front of the other eye.

Fixation of the head of the subject. In order to keep the head of the subject immobile during the experiments, the subject gripped with his teeth round a plate of cautschouc with preformed impressions of his teeth. The plate was fixed to an adjustable metal column, which was in turn fastned to the floor and to the table on which the light adaptation screen was placed.

Experimental distance. All experiments were car-

ried out with the fixation target and the test object placed at a distance of 10.3 m from the subject. Such large distance, where 1 min. of arc corresponds to a linear distance of 3.0 mm, involves several advantages. In the first place it was possible to take readings at different eccentricities within the part of the visual field corresponding to the fovea without a too subtle technique being necessary. In the second place it was so dark at the distance chosen that the reflected light from the face of the subject could be neglected, and a black surface could therefore be considered as physiologically absolutely black, »unilluminated«. Finally it was warrantable to consider this distance, where no accommodation takes place, as infinite in relation to the optical system of the eye.

Fixation target. As fixation target a T-shaped slit illuminated from behind was used. The horizontal part of the slit was 16'40" and the vertical 3', while the width of the slit was 20". The figure was always placed with the short limb lying in the meridian of the visual field which was being examined and pointing in the direction opposite to the test object. On account of this location the fixation target and test object could be rather close to each other (at a distance of 2' to 3') and still appear discrete. The subjects were instructed to fixate the point of intersection of the T-shaped figure.

The slit was illuminated from behind by a 25-watt lamp, the light of which was dimmed by means of a sliding blind and by passing through two plates of frosted glass and being reflected from a sheet of white cardboard placed behind the slit. The light intensity of the fixation target was adjusted so as to be slightly above threshold brightness, so that it could easily be seen by the subject, but was still so weak that no perceptible irradiation could be seen round the figure. During the experiments the fixation target was fastened by means of screws to a special stand, so that it could be placed at any distance and any position required in relation to the test object.

The choice of a T-shaped figure as fixation target was made, because it is presumably easier to fixate a figure con-

sisting of two interseeting lines of a certain length for some time than a point. The latter would probably tend more to cause »gazing blindness« (vide p. 177). In order to find out whether this had any praetical importanee for the experiments on indireet vision, eomparative experiments on the min. separabile at 30' eeeentrieity were carried out with four different test objeets:

a) a luminous square, 40" by 40".

b) a luminous square, 4' by 4'.

e) an unilluminated square, 4' by 4', on luminous ground.

d) a luminous T-shaped slit, 40" wide, eaeh of the limbs being 15' long measured from the point of interseetion.

As to further details of the experiments referenee should be made to page 122. Here it shall only be mentioned that no definite differenee in the applieability of the four objeets was observed.

The illumination of the test object. In the majority of the experiments a luminous test objeet on unilluminated ground or unilluminated objeet on luminous ground was used. As typieal examples of the test objeets the following may be mentioned: two luminous squares on unilluminated ground and two unilluminated squares on luminous ground. A luminous figure on unilluminated ground was prepared by placing a black opaque screen, in which is cut an opening of the required shape, in front of a plate of frosted glass illuminated from behind. As no other light reaches the screen than the extremely faint gleam refleeted from the face of the 10 m distant subject (vide p. 87), it may for a light-adapted eye be considered to be unilluminated. According to the same principle an unilluminated figure on luminous ground was made from opaque material in the required shape pasted on a transparent glass plate placed in front of the frosted glass plate.

While the details of the various test objects and the construction of the apparatus by means of which it was possible to vary the dimensions of the test objects shall be dealt with later, it is here merely the intention to describe the illumina-

ting system by which the light intensity of the frosted glass plate and consequently the brightness of the test object was rendered adjustable (used by *Berger & Buchthal* 1938 a).

A 75-watt lamp (in other cases 150, 500 or 1000-watt lamps) was placed in a housing behind a 12 by 12 cm variable blind with sloping slats, as in a Venetian blind. The inclination of the slats could be altered gradually by moving a pointer outside the box. The deflection of the pointer could be read on a dial. In order to homogenize the light it was passed through filters of frosted glass and thin typewriter paper, so that the frosted glass plate just behind the test object was absolutely uniformly illuminated all over (12 by 24 cm).

In all experiments where it was not the light intensity of the test object which was varied threshold brightness was used for the object, a brightness which made the object just clearly visible for the subject under the experimental conditions used. The aim of using such a small brightness was to minimize the importance of the optical imperfections of the eye, so that the illumination of the parts of the retina on which the image of the unilluminated parts of the object was formed would consequently become subliminal (conf. p. 11). A few experiments (vide pp. 145 and 167) were carried out with the background of the test-object illuminated from before. For this purpose a lamp in a housing screened in the direction of the subject was used. A photographic shutter was mounted in the opening of the lamp housing, so that the illumination could, if required, be switched on momentarily. This shutter was used for some of the experiments on discrimination of thickness of line (vide p. 167). In this case the test object was a dark slit in a white screen illuminated from before. When the screen was illuminated momentarily, it was of course impossible to maintain the fixation of the slit in the dark periods. A small spot of light (abt. 2' in diameter) on the white screen contiguous to the centre of the slit was therefore used as fixation target. The light spot was produced by projecting a narrow pencil of rays from a 3 V lamp on the screen.

The min. separabile was also examined by mo-

mentary illumination with varying exposure time. The test object consisted of two luminous squares on unilluminated ground, for which reason the shutter was placed immediately in front of the squares. In order to maintain the fixation during the unilluminated periods, a fixation target was used consisting in a small white dot painted on the blind of the shutter exactly in front of the intermediate space between the squares. The white dot was illuminated by a narrow pencil of rays thrown sideways and obliquely on the dot. By this method it was attained that, at the moment when the shutter opened, the fixation target disappeared and the test object became visible, the light did not fall upon the unilluminated ground of the luminous squares, but was absorbed by the black inner wall of the shutter frame.

The shutters used were standardized by means of a photo-chronograph with known recording velocity, a light ray being sent through the shutter while it was being released.

Calculation of the illumination of the stimulated retinal area. At threshold brightness the illumination of the test object was adjusted at such a low level that it was impossible to measure it by means of one of the ordinary objective luxmeters (a photo-electric cell connected to a rather sensitive galvanometer, the dial of which records the illumination of the photo-cell in lux). The apparatus was therefore calibrated for various positions of the pointer by means of a photo-electric cell connected to a more sensitive compensation arrangement. The readings were converted to lux by comparison with the deflection on an objective luxmeter, when the illumination was adjusted at its maximum.

The illumination (B) of the retinal images was calculated from the formula: $B = \frac{a^2 \cdot L \cdot \frac{\pi}{4} \cdot p^2}{x^2 \cdot \sigma^2}$ (*Berger & Buchthal* 1938 a) where a equals the distance of the photo-electric cell from the luminous plate during the reading, L the number of lux measured, p the diameter of the artificial pupil, x the distance from the subject's eye to the test object (in mm), while σ^2 re-

presents the stimulated retinal area in sq. mm. The loss of light caused by the passage through the refractive media of the eye is neglected.

B. Special Arrangements for Examining Individual Visual Functions.

1. Arrangements for Examining the Physiological Resolving Power.

a. The minimum separabile.

The test objects used were either two luminous squares on unilluminated ground or two unilluminated squares on luminous ground.

Luminous squares on unilluminated ground.

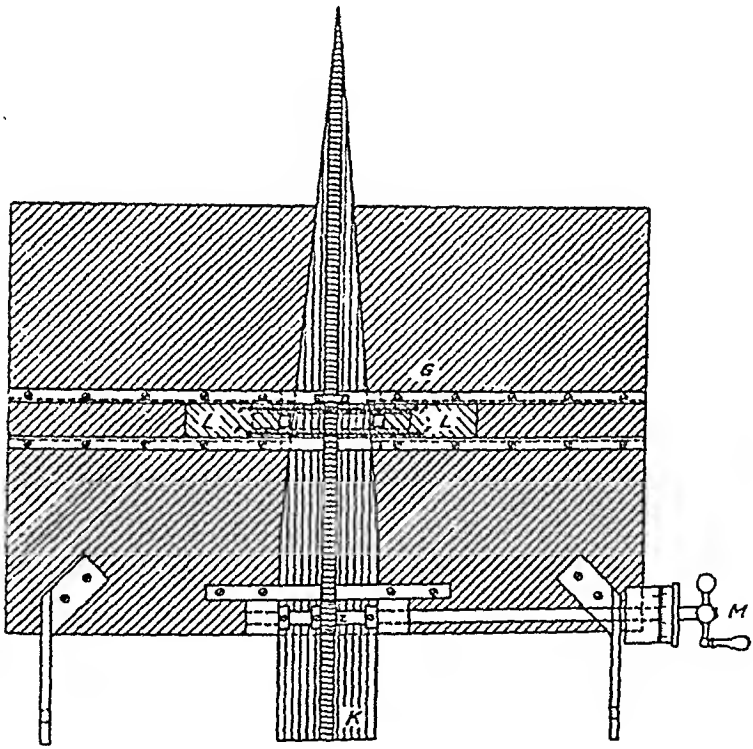


Fig. 4.

Wedge-arrangement for measuring the min. separabile for luminous squares on unilluminated ground (range of measurements 2.7 to 50 mm). The illustration shows a rear view of the apparatus. L and L are the exchangeable metal discs with square apertures. These metal plates slide on rails on the plate G and are kept apart by the wedge-shaped metal plate K. They are held tightly against the latter by means of springs F. The wedge is moved by means of the micrometer screw M, the spindle of which is provided with a gear Z engaging with a rack on the wedge.

The luminous squares (vide fig. 4) were cut out of two small brass plates (L) which were illuminated from behind. These plates, which were blackened both on the surface and in the cutting, could slide on a pair of rails placed on a large fenestrated brass plate (G) and could easily be replaced by other corresponding plates with apertures of different sizes. Two sets of plates had circular apertures with diameters of 0.5 and 1.0 mm, others had square apertures, the sides of which were either 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 7.0, 9.0, 11.0 or 14.0 mm. At the observation distance used the diameter of the smallest apertures corresponded to an angular diameter of 10" and the length of the side of the largest squares to 4'40".

By means of a wedge-shaped brass plate (K), which could be moved up and down by means of a rack and gear (Z), the distance between the squares could be varied from 2.7 mm to 50 mm (54" to 16'40") and the position be read with an accuracy of 0.1 mm (2") on the micrometer screw M. The two small brass plates (L) were held so tightly against the brass wedge by means of two helical springs that no light could penetrate between plates and wedge.

Besides the apparatus described, which is the same as the one used by *Berger* (1936) a similar one with a finer wedge *(Berger & Buchthal* 1938 a), which allowed for a distance between the squares varying from 1.5 to 17 mm (30" to 5'40"), was also used. The accuracy of the reading was the same, 0.1 mm.

On investigating the dependence of the min. separabile on the size of the square it proved to be desirable to extend the investigation to squares considerably larger than those belonging to the apparatus with the wedge. The min. separabile for such large squares is, however, so small that it is technically impossible to construct an apparatus on the wedge principle, by means of which such large luminous squares can be approached to each other, so that they are seen in cohesion. It was therefore necessary to apply a principle according to which the variation of the distance between the squares was discontinuous. The large squares with a length of side of 20 to 120 mm (6'40" to 40') were made by placing an oxydized

copper wire in front of a luminous rectangle of such dimensions that the wire divided the rectangle in two luminous squares. When the wire was replaced by other wires of progressively increasing or decreasing thickness, the intermediate space between the squares was altered correspondingly.

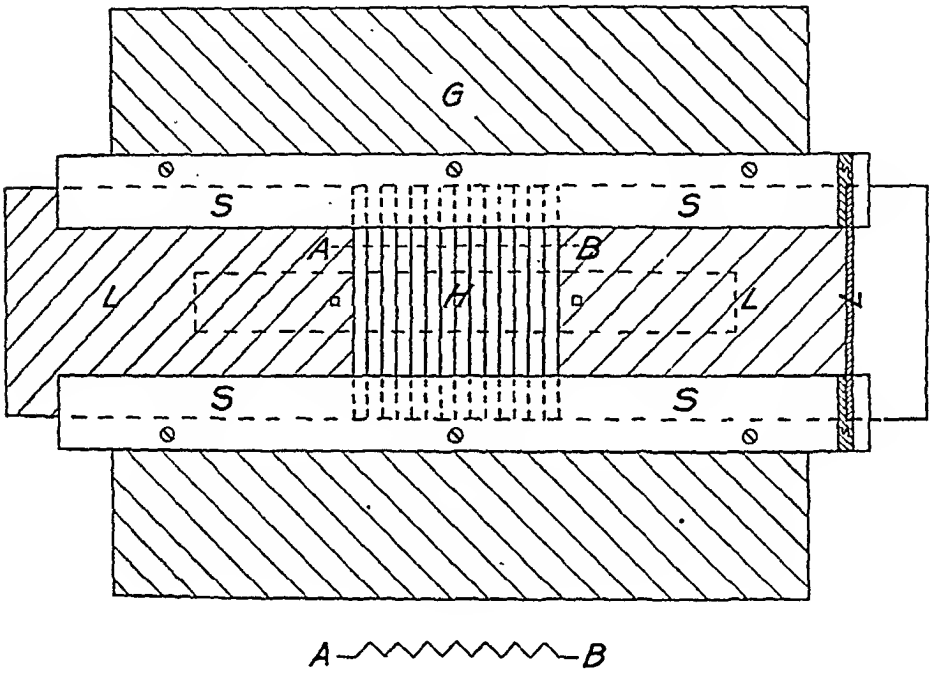


Fig. 5.

Apparatus for measuring the min. separabile for luminous squares on unilluminated ground with an intermediate space varying from 32 to 180 mm. Two rails (S) are fastened by screws to the plate G. Two plates L, each with a square aperture, slide on the rails. The space between the plates is enclosed by an „accordion" H of black paper.

The thickness of the wires used varied from 0.035 mm to 2.0 mm, angular diameter 0.7", 2", 4", 6", 8" and so on up to 40".

The wedge arrangement described also proved insufficient for measurements of the min. separabile by indirect vision, where it was necessary to have a very large distance between the squares. Two other apparatuses were therefore constructed, each for a certain range of measurements. The first of these (fig. 5) consisted in a large plate (G) with an oblong hole along which two rails were placed. On these rails two smaller plates (L) with square apertures (40" by 40") could be slid

by hand. The plates were connected by an »accordion« (H) of black paper, which prevented light from reaching the space between the plates. This device allowed for a variation of the distance from 32 to 180 mm (11' to 60'). The readings were carried out by means of a rule graduated in millimetres.

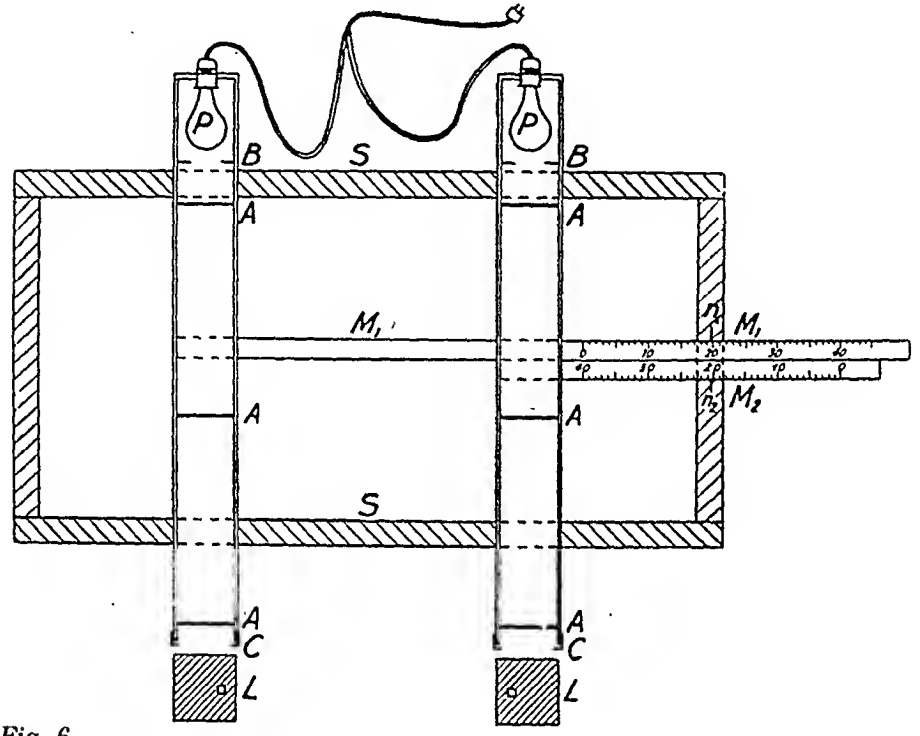


Fig. 6.

Apparatus for measuring very large values of the min. separabile for luminous squares on unilluminated ground (range of measurements 3 to 100 cm).

L represent transparent glass plates, on which black paper with a square aperture is pasted. The glass plates can each be placed in front of a lamp housing in the groove C. The light from the lamps P is reduced by means of the sliding blinds B and homogenized by the frosted glass plates A before it reaches the glass plates with the squares. While being kept parallel to each other the two long boxes may be displaced by hand on the rails S by means of the wooden rods M1 and M2, on which the distance between the boxes may be read at the marks n1 and n2.

In the other apparatus (fig. 6) each square was provided with a lamp housing, so that the distance between the squares could be increased considerably. The squares consisted in square »windows« in glass plates (L) elsewhere covered with black paper. The length of the sides of the squares varied from 0.5 to 20 mm (10" to 6'40"). The glass plates with the squares were all of the same size namely 9 by 12 cm. Each plate could be placed in front of its lamp housing. The latter

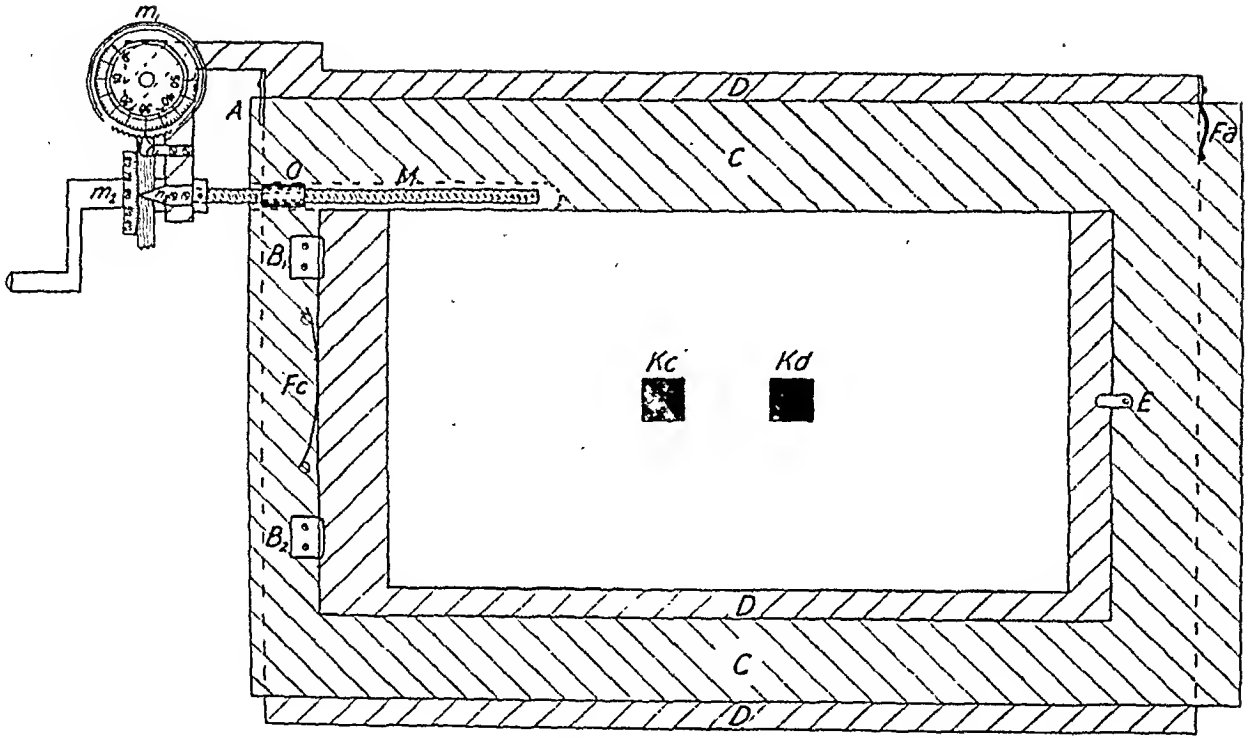


Fig. 7.

Apparatus for measuring the min. separable for unilluminated squares on luminous ground. A transparent glass plate with the square K_d is inserted in the metal frame D and supported by the plate C. By the spring F_a the glass plate is held against the stop A. Another glass plate with the square K_c is also placed in a frame and supported by the part of the frame D which is inside C, it is located by the spring F_c and the small plates B_1, B_2 and E. The frame C can be displaced in relation to D by means of the micrometer screw M, the spindle of which is threaded and passes through a nut with 1 mm pitch. The nut is soldered to C. The dial m_1 reads whole millimetres, while the rule m_2 reads centesimal parts of a milimetre, the transmission from M being effected by a thread which makes m_1 travel a distance equal to one graduation for each revolution of the spindle. The reading takes place by means of the pointers n_1 and n_2.

was a narrow, closed box, 90 cm long, at one end of which the glass plate with the square was attached, while the other end was provided with a lamp holder with a lamp P (of 25 or 100 watt). The light from this lamp was controlled by means of a simple sliding blind, and homogenized by passing through three frosted glass filters and reflected from the white-painted walls of the box. The two long boxes could be moved parallel to each other along a pair of rails (S). The distance between the squares in mm could be read on a rule placed on two wooden rods M_1 and M_2, by means of which the boxes were moved. With this apparatus the space between the squares could be varied from 3 to 100 cm (10' to 5°33').

Unilluminated squares on luminous ground. The unilluminated squares on luminous ground were produced by pasting black, intransparent paper on transparent glass plates, one square on each plate. Pairs of glass plates with squares having the following lengths of sides were at hand: 2 mm, 3, 4, 5, 7, 10, 15, 20, 25, 30 and 45 mm (40" to 15'). Each pair of glass plates could be placed close to each other (vide fig. 7) in two metal frames C and D, of which C was slidable in relation to D by means of the micrometer screw M. The accuracy with which the reading took place was 0.01 mm. The squares could be approached so as to touch each other and be removed to a distance of 35 mm from each other. It was easy to carry out the zero adjustment. When the glass plates were illuminated from behind the squares appeared unilluminated on a homogenous, luminous ground, 17.5 by 23.5 cm (58' by 78'). The arrangement is the same as the one used by *Berger* (1939). In all experiments where the illumination of the object was kept constant, threshold brightness was — as previously mentioned — used for the background of the squares.

b. Perception of movement.

The test object (vide fig. 8) used for the experiments on perception of movement was a square aperture, 1.5 by 1.5 mm (30" by 30"), illuminated from behind, in an oblong, blackened metal plate which could be moved sideways on two rails (S)

by a crank mechanism (K). The latter was driven by an electromotor, the speed of rotation of which could be varied by means of a rheostat inserted in the circuit.

The distance travelled by the metal plates (and consequently by the square) could be altered by stages of 1 mm

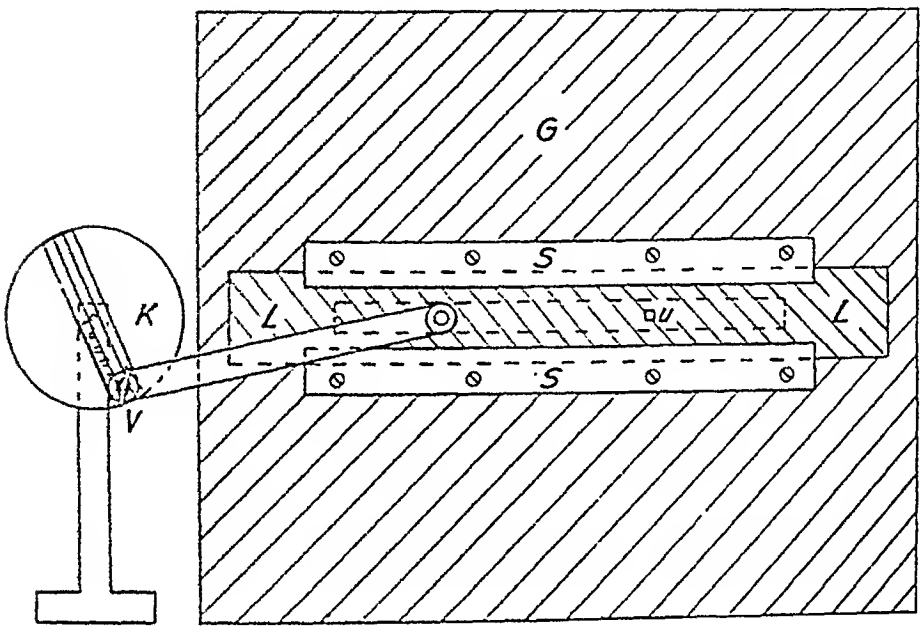


Fig. 8.

Arrangement for measuring the smallest recognizable movement. On the plate G two rails (S) are fastened by means of screws. An oblong metal plate L with a square aperture slides along these rails. L is moved by the crank mechanism K. The amplitude may be regulated by slackening the wing nut V and altering its position.

(20"). A revolution counter incorporated in the apparatus made it an easy matter to find the frequency of the movement by means of a stop watch. When the whole apparatus was placed in front of the illuminating system described on p. 89, the subject saw the square as a luminous, moving point on an unilluminated ground.

At the experiments a frequency of 2 or 3 (excursions to both sides) per sec. was used. With regard to the reasons justifying the choice of this particular frequency reference should be made to p. 149.

c. Form sense.

The test objects used for the investigations on form sense were either a luminous square on unilluminated ground or a corresponding equilateral triangle. The size of the two figures was variable, as each of them represented the aperture in a blind illuminated from behind and

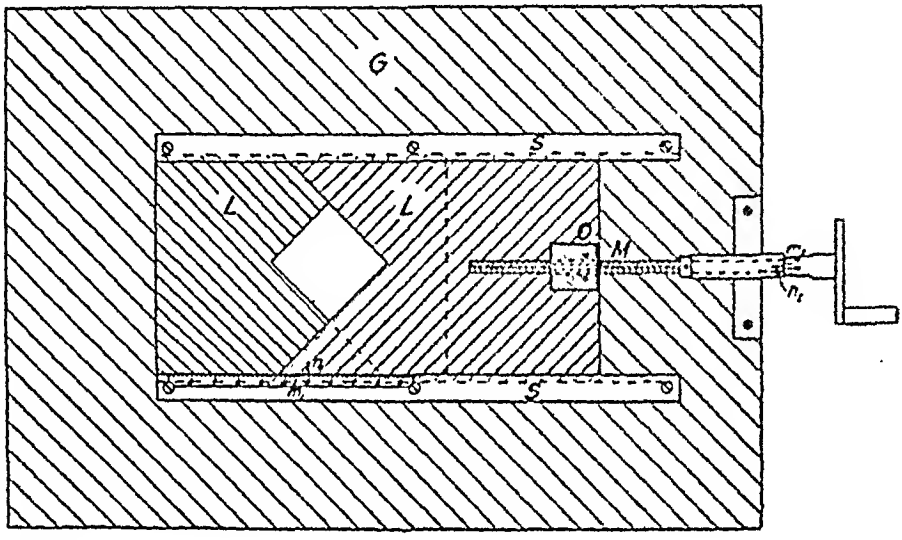


Fig. 9.

Blind with variable square aperture for experiments on form sense.
The two plates L have each a right-angled aperture which together form a square. While the left-hand plate on the figure is rigidly connected to the bed plate G, the right-hand one is made slidable along the rails S by means of the micrometer screw M and the nut O. The length of the diagonal of the square can be read at n_1 on the rule m_1, which reads whole millimetres, and at n_2 on the rule m_2, which reads tenths of millimetres.

of a design similar to that of the so-called *Foerster*'s blind. (*Aubert*, 1865 [44]). In the case of the square the blind (vide fig. 9) consisted of two thin vulcanite plates (L), each with an aperture in the shape of an isosceles, right-angled triangle. The size of the square formed by the two triangles could be varied by displacing one triangle along the diagonal of the square. This was carried out by means of a screw mechanism. The maximum length of the diagonal was 84 mm (28'). The accuracy with which the reading took place was 0.1 mm. With another and larger blind made from metal the accuracy was not so good (0.5 mm), but the range of measurements larger,

as the maximum diagonal was 144 mm (48'). By replacing the two thin plates of the last mentioned blind by another set, of which one is cut off straight, while the other has an aperture with an angle of 60°, the test object was converted into an equilateral triangle. In that case the distance measured was the perpendicular on one of the sides.

Test objects of threshold brightness were also normally used for the experiments on form sense.

2. Arrangements for Experiments on Complex Functions.

a. Aligning power.

The test object used for the experiments on aligning power was a luminous slit, 1 mm (20") or 2.5 mm (50") wide and 110 mm (37') long, of threshold brightness on unilluminated ground. The slit was produced by pasting black paper on two glass plates, so that each plate showed one half of the slit. The plates fitted into the same metal frame which was used for the experiments on the min. separabile for unilluminated squares (vide p. 96). The two half parts of the slit could thus be displaced with an accuracy of 0.01 mm. The illuminating system was the same as the one described on p. 89.

b. Discrimination of thickness of line.

The experiments on discrimination of thickness of line were carried out with a luminous slit of threshold brightness in an unilluminated background as well as with a dark slit in a white ground illuminated from before.

The apparatus for the experiment consisted of a large vulcanite plate (vide fig. 10) with a long, rectangular slit. Adjoining one of the longitudinal sides of the latter were two small vulcanite plates (L), which could each be displaced by means of a micrometer screw (M), so that the width of each half of the slit could be varied with an accuracy of 0.1 mm (2"). The length of the slit was also adjustable by means of the two slidable plates (C). The maximum length of each half of the slit was 55 mm (18'), the maximum width 30 mm (10'). While one side of the apparatus was black, the other was

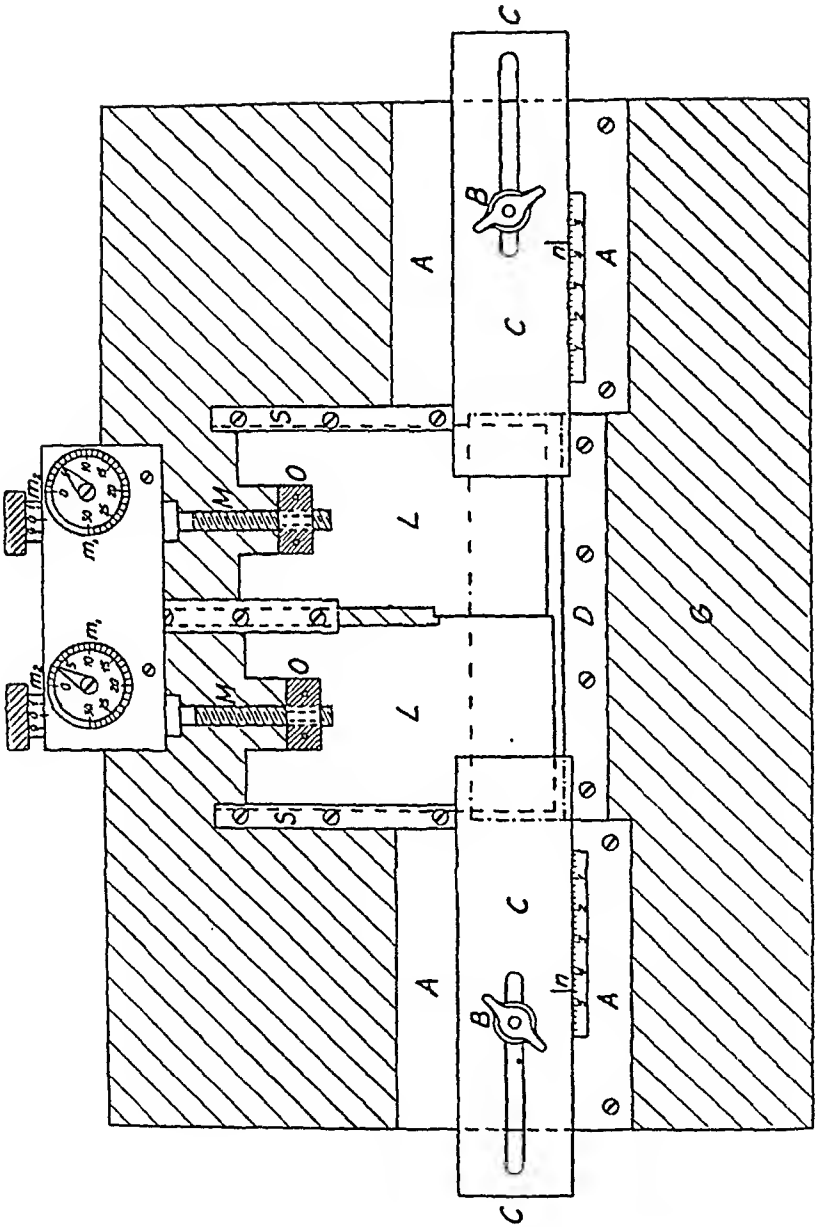


Fig. 10.

Apparatus for experiments on discrimination of thickness of line.
In the bed plate G a slit has been cut. One side of the slit is formed by the rail D, the others are shown by the dash and dot line (— . — . —). The ends of the slit may be screened by the plates C, which are guided by the rails A and kept in place by the wing nuts B. The length of the slit can be read at n on a rule on the rail A. The two halves of one longitudinal side of the slit are covered by the plates L, by displacing the latter vertically the width of the slit may be altered. The plates L, which slide in rails S, are moved by means of the micrometer screws M through the nuts O. The width of the slit can be seen from the dial m_1, which reads whole millimetres, and from the graduation m_2 on the spindle of the micrometer screw, which reads tenths of a millimetre.

covered by white drawing paper. In this way the slit could be used both as a luminous slit and as a dark slit in white ground. In the latter case a wooden box lined with black, woollen material was placed behind the object, so that the slit was, as far as possible, »absolutely black«. The illumination, when from before, came from a lamp in a housing placed at some distance from the apparatus, vide p. 89.

C. Course of Experiments and Calculation of Results.

None of the subjects had been acquainted with the problem which the experiments were intended to elucidate. Even after a series of measurements the subject could only vaguely indicate the direction in which the results of the experiments pointed, as the conformity between the numerical results and the subject's impression was very rough.

In every experiment two thresholds were determined in alternate order for the function examined. By always determining two thresholds alternatively the less well defined range between the two thresholds was passed through before every measurement. The definitions of the thresholds read appear from table V (vide next page).

The movement of the squares when measuring the min. separabile and also the variation of the other test objects was always carried out so slowly that the subject had sufficient time to scan the object before — by rapping a signal — he had to register, that the expected threshold had been reached. In some of the experiments a momentary illumination of the test object was, as previously mentioned, used, and the variable dimension was consequently varied by steps. As the apparatus was operated in semi-darkness, the micrometer screw was not set at definite figures, but turned so far each time that the range between the two thresholds was divided in three to seven steps. Every time the shutter had been

Table V.

Definitions of the Thresholds Read.

1. The Physiological Resolving Power.

Function.	Threshold I.	Threshold II.
a. The min. separabile:	The minimum distance separating the squares when diverging, that makes them appear clearly discrete.	The largest angular distance between the squares at which they are seen to be contiguous.
b. Perception of movement.	The smallest recognizable movement of the luminous point synchronous with the actual movement of the point. (By this definition it is avoided that the subject registers apparent movements.)	The largest movement of the luminous point which cannot be recognized.
c. Form sense.	The minimum length of side at which it is possible to recognize the angularity of the polygon.	The largest length of side at which it is just impossible to recognize the angularity of the polygon.

2. Complex Functions.

Function.	Threshold I.	Threshold II.
a. Aligning power.	The minimum displacement of one half of the slit in relation to the other which can be clearly recognized, »displacement threshold«.	The largest displacement of the two halves of the slit by which they seem to be in alignment with each other.
b. Discrimination of thickness of line.	The minimum discernible increase in the width of one half of the slit.	The smallest observable decrease in the thickness of the same half of the slit.

released, i. e. for each step, the subject stated by a signal whether the threshold in question had been reached.

In nearly all of the experiments 4 determinations of each of the two thresholds were carried out in alternate order. An essential reason why not more than 8 measurements were included in each series was the fact that the dispersion of the values of the thresholds increases with the number of individual readings, when this exceeds 5 to 8 (*Berger* 1936). The phenomenon is probably due to fatigue.

During each sitting, lasting about one hour, 8 to 10 series of readings could be carried out, each followed by a small pause, during which the subject could rest, while alterations in the arrangement were carried out.

When the influence of a certain factor on the function was to be examined, e. g. the influence of the retinal eccentricity on the min. separabile, the factor was altered for each series. so that — in the instance mentioned — both thresholds were determined at 8 to 10 eccentricities.

In order to reduce the effects of fatigue, if any, on the results, all experiments were repeated on another day taking the series in the opposite order, and then the average of each two corresponding series was calculated. The average values, each based on 8 readings, were taken as the experimental results proper, and a curve was plotted with the function examined as ordinate and the variable factor as abscissa.

In order to obtain a view of the accuracy of the experiments separate investigations on the accuracy of the threshold determinations with calculation of the standard deviation and variation coefficient were carried out for each test object. In this case it was necessary to carry out a larger number of measurements under constant experimental conditions in order to obtain a number of figures sufficient for the calculation. (10 measurements of each threshold divided in two series of 5 each, and the same experiment repeated in the reverse order on another day).

The variations of the difference between the ordinates for

threshold I and threshold II could also be regarded as a measure of the accuracy, as the curves representing the two thresholds in some cases ran parallel to each other (vide e. g. fig. 17, p. 130), in other cases the slopes of the two curves were different although of the same tendency (vide fig. 11, p. 107).

During the experiments it was often observed that the level of the thresholds shifted slightly from day to day, as also shown by *Berger* (1935) and *Craik* (1938). Such deviations, which are usually met with in sense-physiological experiments, *(v. Kries* 1923[144]), may be explained partly by variations in the excitability of the sense organ and partly by the subject unconsciously altering his choice of threshold slightly from one day to another. On account of these day-to-day variations the absolute values can only be compared directly with a certain reservation.

Chapter IV

Experiments on the Physiological Resolving Power 1

EXPERIMENTS ON THE MINIMUM SEPARABILE

A. The minimum separabile for Luminous Points on Unilluminated Ground at Different Retinal Eccentricities

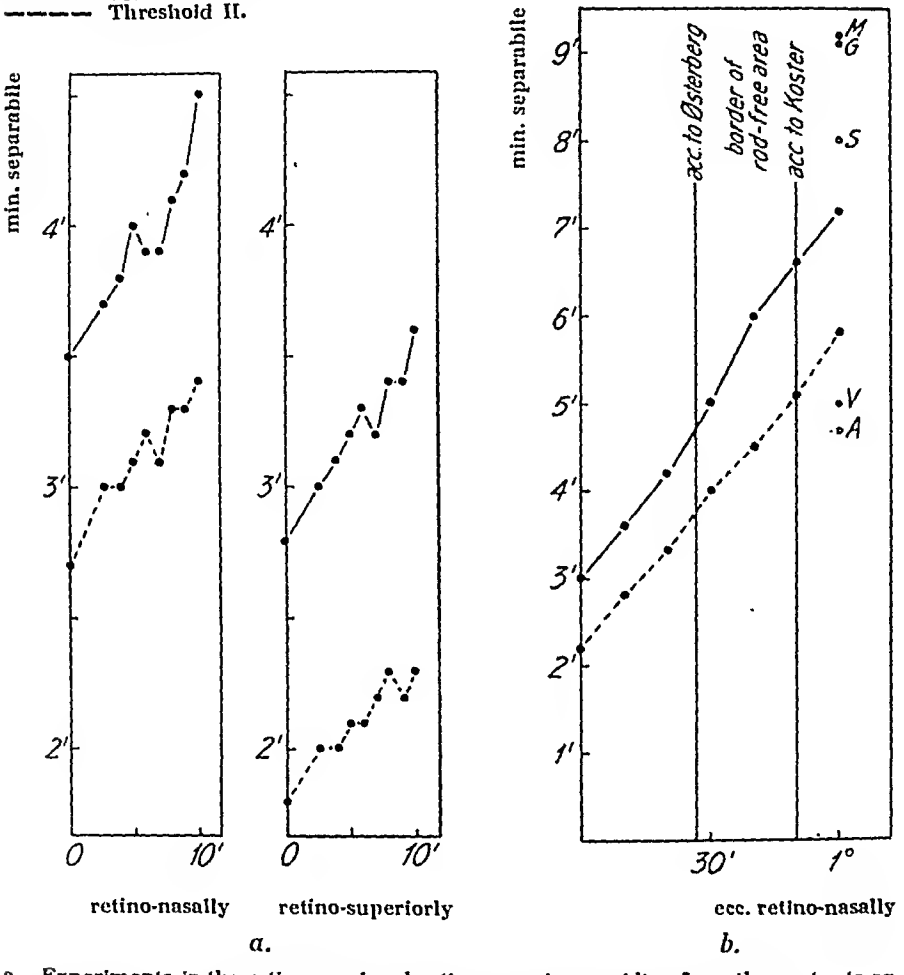
As already mentioned on pp. 64 and 78 the aim of the experiments was to determine the min. separabile by central and indirect vision in order thereby to obtain a measurement of the diameter of the functional units. The test objects were luminous squares, 40 by 40 sec. of arc, on unilluminated ground. On account of the small visual angle the squares appeared to the subject as luminous points.

The smallest eccentricity at which the min. separabile could be examined was 2'40", as it was impossible to see the squares separated from the fixation target, when they were placed closer to it. Four series of experiments were performed within four ranges of eccentricity, namely: 0—10' retino-nasally and retino-superiorly, 0—60', 0—5°, and 0—10° retino-nasally. During the experiments in the range from the centre to an eccentricity of 10' the squares were placed so that a line connecting them was perpendicular to the meridian examined. In this way the eccentricity of the squares was maintained, when their relative distance was altered during the experiments. At eccentricities larger than 10' both squares were placed in the meridian in question, as this was technically most convenient with the apparatus employed, and the position of the squares in relation to the meridian proved to be of no importance to the result of the experiment (vide p. 118).

The min. separabile by central vision was found to be between 2.5' and 4' (vide fig. 11) in conformity with the values found by *Aubert* (1865 [210]), *du Bois Reymond* (1886), *Wertheim* (1887), *van Heuven* (1937)

Fig. 11.

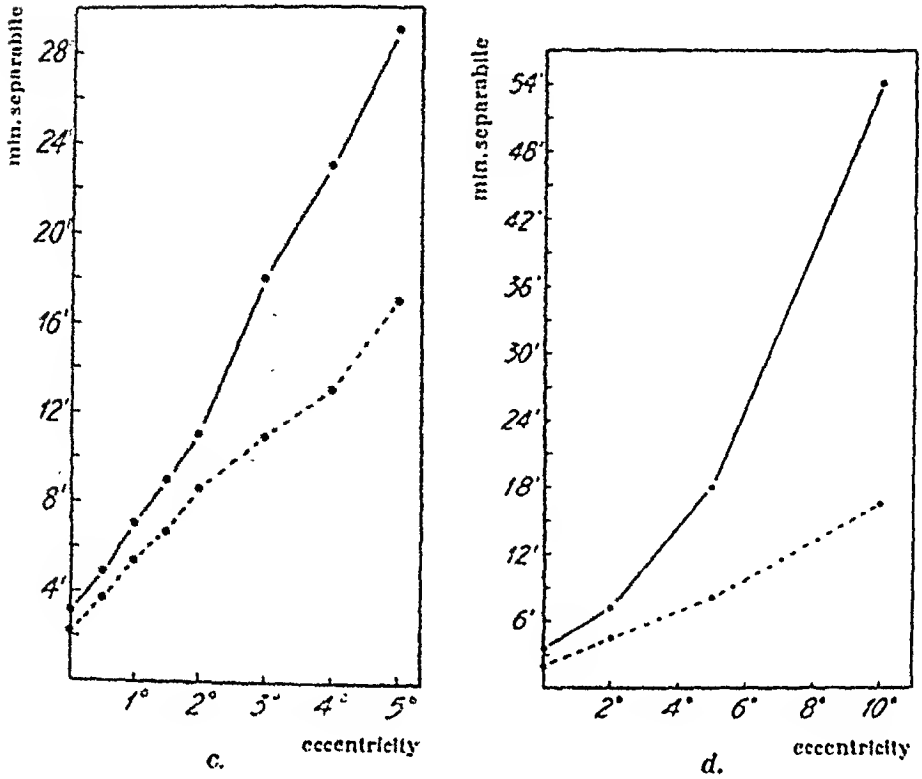
The minimum separabile for luminous squares on unilluminated ground at various retinal eccentricities.
Abscissa: Eccentricity in min. of arc or degrees.
Ordinate: Min separabile in min. of arc.
——— Threshold I.
– – – – Threshold II.



a. Experiments in the retino-nasal and retino-superior meridian from the centre to an eccentricity of 10°. Average of results from 3 subjects (M, A, S and A, V, S).

b. Experiments from 0 to 1° retino-nasally. The points of the curve represent the average values for 5 subjects (M, G, S, V, A). At an eccentricity of 1° threshold I for each subject has been recorded. Moreover the extent of the rod-free area (according to *Østerberg* (1935 [64]) and *Koster* (1895)) has been marked off.

and *Berger & Buchthal* (1938 a) with luminous points or very small squares (conf. p. 21). As the experiments were performed with squares of threshold brightness, the min. separabile determined was smaller than by any other brightness (vide p. 126) and must be assumed to be a function of the mean diameter



c. Experiments from 0 to 5° retino-nasally. Average for 4 subjects (G, M, S, V).

d. Experiments from 0 to 10° retinonasally. Average for 3 subjects (A, V, S).

of the functional units. In the foveal centre it must thus be abt. 3 min. of arc or abt. 15μ. If it is assumed that the diameter of the cones here is 2.6 μ (according to *Østerberg*, vide p. 16) then the functional units in the foveal centre must each consist of several cones. The diameter of such a group may be estimated at abt. 6 times the width of a cone.

By all readings outside the centre of the visual field, even the one at 2'40" from the fixation point, the min. separabile was found to be larger than in the centre (vide fig. 11 a). In the whole area examined up to an eccentricity of 10° the min. separabile increased uniformly with the distance from the fixation target. The steepness of the slope of the curve was some-

what different for the various subjects, most pronounced for G, M, S, less so for A and V. (vide fig. 11 b).

The boundary between central and indirect vision in the visual field or on the retina is quite naturally drawn at the eccentricity at which the min. separabile exceeds its central minimum value. It appears from the experiments both in the horizontal and in the vertical meridian that central vision must be confined to a retinal area with a radius less than 2'40". The extent of central vision is thus of the same size as the retinal fixation area, whether this is determined objectively or subjectively (conf. pp. 57 and 58).

It might have been expected that structural boundaries in the retina, especially the border of the rod-free area, had appeared as breaks in the curves. (Confer the fact that *Purkinje's* phenomenon cannot be produced at eccentricities smaller than 40' to 50', eccentricities exactly corresponding to the border of the rod-free area (44' eccentricity) (e. g. *Dieter* 1924). Confer further that *Adler & Meyer* (1935) found that the discrimination of thickness of line is constant within a »physiological fovea« extending to an eccentricity of 42'). As previously mentioned the min. separabile on the contrary increases quite uniformly with the eccentricity. This confirms the results found by *Wertheim* (1887) and *Weymouth* (1928), (vide p. 63), irrespective of the fact that the experimental conditions of these authors cannot be directly compared with those of the present investigation. An attempt to explain the very even course of the curve on basis of the retinal structure shows that there are several possibilities (conf. p. 68). In the first place the number of cones per unit of area may play a role. For one thing the diameter of the cones increases peripherally in the fovea, and their relative distance increases moreover when the rods appear. In the second place the number of cones connected to the same optic nerve fibre increases from a certain eccentricity, and it is finally possible that outside a certain area the units are not functionally separated.

From the value of the min. separabile by central vision

Table VI.

Østerberg's countings in the temporal retinal meridian.

Eccentricity	Number of cones per 100 μ	Calculated width of cones or distance between the centres of two neighbouring cones	
0	38.4	2.6 μ	32"
10.8'	32.4	3.1 -	38"
16.6'	29.1	3.4 -	41"
17.4'	27.4	3.7 -	45"
26.4' *Rods appear.*	23.7	4.2 -	51"
43.2'	22.3	4.5 -	54"
55.2'	20.3	4.9 -	59"
1°34'	17.1	5.9 -	72"
2°42'	15.2	6.6 -	80"
4°	12.3	8.1 -	98"
17°42'	7.4	13.5 -	163"

the width of the functional units in the centre was calculated at abt. 6 times the width of a cone. If it is assumed that the functional units everywhere consist of the same number of cones, then the diameter of the units in the fovea may be calculated at 6 times the diameter of the cones and outside the rod-free area at 6 times the distance between the cones which prevails at the eccentricity in question. In table VI the width of the cones and the distance between the cones respectively are calculated for various eccentricities on basis of *Østerberg's* (1935 [64]) countings of the number of cones per unit of area.

On fig. 12 the calculated diameter of the functional units is compared with measurements of the min. separabile in the same retinal meridian. It appears from the figure that the two curves run parallel up to the border of the fovea and from here diverge. It thus seems as if the functional units everywhere in the fovea consist of the same number of cones per optic nerve fibre, while outside the fovea they incorporate more cones, or it is possible that they are no more functionally separated from each

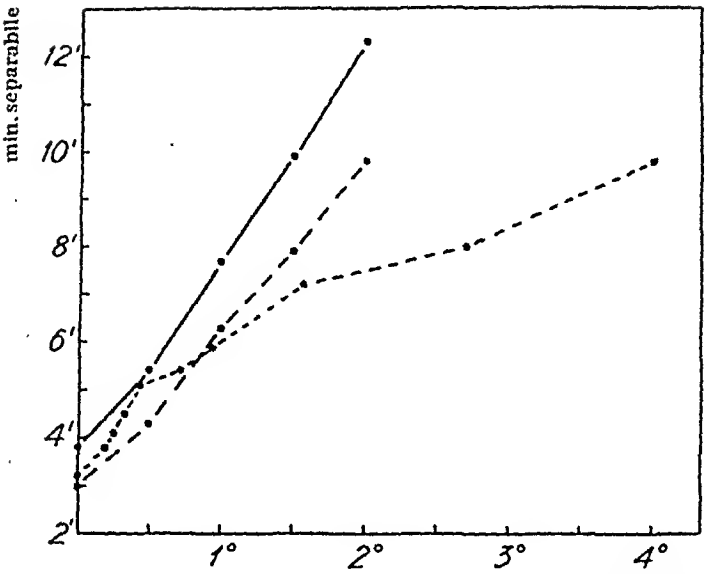


Fig. 12.

The min. separable for luminous squares on unilluminated ground up to a retino-temporal eccentricity of 2°. Average for 3 subjects (G, M, S). On the graph a curve is plotted showing the diameter of the functional units calculated from the countings of the number of cones per unit of area performed by *Osterberg* (1935 [44]) on the assumption that the diameter of the functional units is always — as in the foveal centre — equal to 6 times the width of the cones or the distance between the centres of two neighbouring cones respectively.
Abscissa: Eccentricity in degrees.
Ordinate: Min. separable in min. of arc.
———— Threshold I.
— — — Threshold II.
· · · · · · The calculated diameter of the functional units at the eccentricity in question.

other on account of a dispersion of the impulse (conf. p. 71).

The fact that the distance between threshold I and threshold II (vide fig. 11) increases with increasing eccentricity can probably be explained by the gradually increasing difficulty with which the subject judges whether the squares are separated or not.

The accuracy of the experiments.

Separate experiments were performed on the reliability of the technique used for the investigations on indirect vision and also in order to establish a basis for a discussion of the results.

The experiments consist in series of determinations of the thresholds carried out by central vision and by an eccentricity

of 30', 1°, 1°30', and 2°, others at 5° and 10° and, finally, for one subject (V) at an eccentricity of 15°. In order to obtain a number of readings so large, that a reckoning of the experimental error may be based on it, series consisting of 5 determinations of each threshold were carried out, and each series was repeated after a short pause. By repeating the whole experiment on another day with the series in the reverse order a total of 20 threshold determinations under the same experimental conditions was obtained.

The dispersion of the threshold determinations on both days together was taken to indicate the accuracy of the experiments. The calculation was performed in the following manner:

The dispersion (S) of the threshold determinations of all series from both days is calculated from the formula:

$$S = \sqrt{\frac{p_1 \cdot \sigma_1^2 + p_2 \cdot \sigma_2^2}{n - r}}$$

where p_1 and p_2 are the numbers of measurements in each series (i. e. 10), σ_1 and σ_2 the standard deviation of both series, n the total number of readings (20) and r the number of series (2).

In order to express the dispersion in relation to the dispersion range of the mean values a coefficient of variation, K, is calculated from the formula:

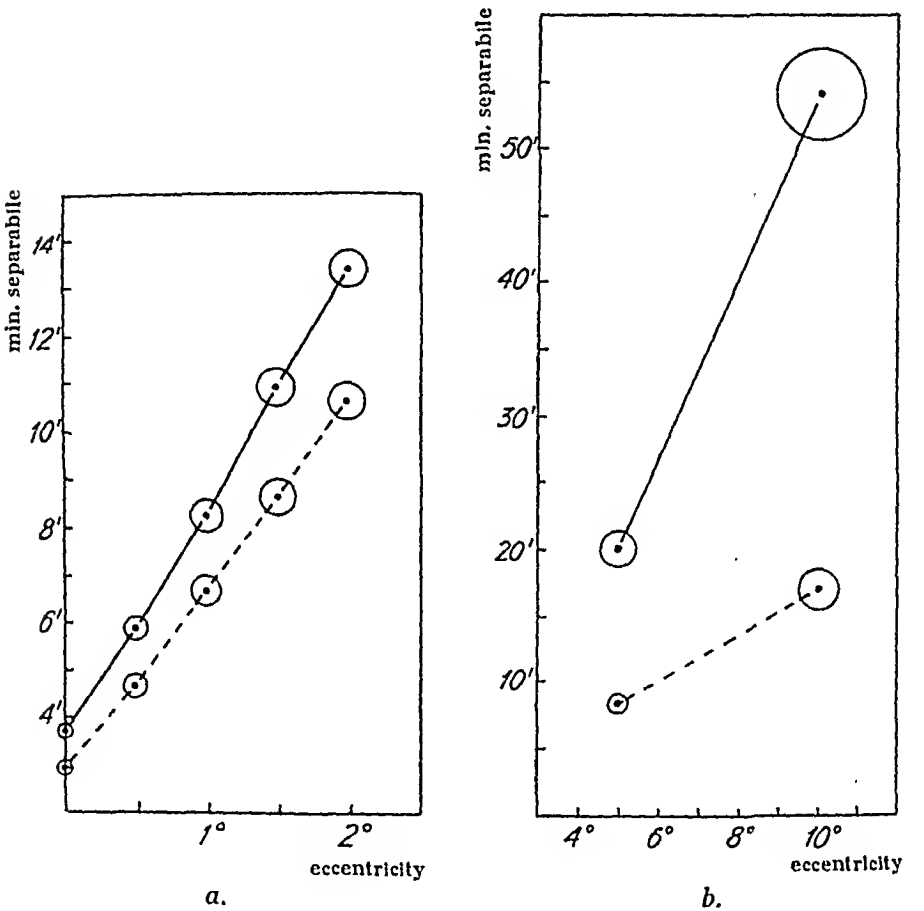
$$K = \sqrt{\frac{p_1 \cdot K_1^2 + p_2 \cdot K_2^2}{n - r}}$$

where K_1 and K_2 are the variation coefficients for the mean values m_1 and m_2 of the min. separabile for each of the two days (e. g. $K_1 = \frac{\sigma_1 \cdot 100}{m_1}$), while the other letters denote the same things as in the above formula.

The results of the experiments, i. e. the mean values of the min. separabile, the calculated dispersion S and the variation coefficient K appear from the table VII. In fig. 13 the mean values of the min. separabile has been plotted against

Fig. 13.

The minimum separabile for luminous squares on unilluminated ground at various retinal eccentricities. *Experiments on the accuracy of the measurements.* The radii of the circles drawn round the points of the curve represent the dispersion of the threshold values, measured in minutes of arc.
Abscissa: Eccentricity, retino-nasally, in degrees.
Ordinate: The min. separabile in min. of arc.
——— Threshold I. — — — Threshold II.



a. Experiments in the range from 0 to 2° of eccentricity. Average values for 3 subjects (G, M, S).

b. Experiments at an eccentricity from 5° to 10°. Average values for 2 subjects (A and S).

the eccentricity. Round each point of the curve a circle has been drawn, the radius of which is the dispersion in minutes of arc.

It appears from the table and from fig. 13 a that within the area from the centre to an eccentricity of 2° the dispersion S increases with increasing distance from the centre (on an average from

Table VII.

Dispersion and variation coefficient in experiments on the minimum separabile at various eccentricities.

a. 0—2° retino-nasal eccentricity.

The minimum separabile (average value for two days).

Threshold I	Subject	0	30'	1°	1°30'	2°	Number of readings
	G.	4.6'	6.5'	9.9'	12.7'	14.9'	20
	M.	3.5'	6.1'	7.3'	9.3'	11.4'	20
	S.	3.0'	5.2'	7.7'	10.9'	14.1'	20
Average		3.7'	5.9'	8.3'	10.9'	13.4'	60
Threshold II	G.	3.3'	4.5'	7.3'	9.6'	11.5'	20
	M.	3.1'	5.6'	6.7'	8.4'	10.5'	20
	S.	2.4'	4.0'	6.0'	8.0'	9.9'	20
Average		2.9'	4.7'	6.7'	8.6'	10.3'	60

Dispersion (S) of threshold values.

Threshold I	G.	0.22'	0.35'	0.67'	0.52'	0.32'	20
	M.	0.14'	0.17'	0.29'	0.42'	0.43'	20
	S.	0.10'	0.21'	0.18'	0.30'	0.48'	20
Average		0.15'	0.24'	0.38'	0.41'	0.41'	60
Threshold II	G.	0.17'	0.36'	0.48'	0.66'	0.35'	20
	M.	0.10'	0.20'	0.30'	0.33'	0.27'	20
	S.	0.12'	0.20'	0.15'	0.16'	0.44'	20
Average		0.13'	0.25'	0.31'	0.38'	0.35'	60

Variation coefficient (K).

Threshold I	G.	5.1	5.7	6.8	4.2	2.1	20
	M.	4.0	2.8	3.9	4.6	3.5	20
	S.	3.1	4.0	2.3	2.7	3.4	20
Average		4.1	4.2	4.3	3.8	3.0	60
Threshold II	G.	5.4	8.7	6.7	3.7	3.8	20
	M.	3.3	3.6	4.5	4.0	2.4	20
	S.	5.2	5.2	2.6	2.0	4.3	20
Average		4.6	5.8	4.6	3.2	3.5	60

b. Eccentricity 5°—15°, (Subject A and S retino-nasally, subject V retino-temporally).

The minimum separabile (Average value for two days).

	Subject	5°	10°	15°	Number of readings
Threshold I	A.	6.3'	29'		20
	V.	7.2'	9.5'	22'	20
	S.	34'	79'		20
Threshold II	A.	4.7'	14'		20
	V.	3.6'	5.7'		20
	S.	12'	19'		20

Dispersion (S) of threshold values.

Threshold I	A.	0.34'	1.60'		20
	V.	1.58'	2.31'	6.8'	20
	S.	2.45'	5.3'		20
Threshold II	A.	0.31'	0.9'		20
	V.	1.54'	1.4'		20
	S.	1.11'	2.2'		20

Variation coefficient (K).

Threshold I	A.	4.7	5.5		20
	V.	6.6	6.0	9.7	20
	S.	7.7	6.8		20
Threshold II	A.	6.8	6.5		20
	V.	11.9	6.8		20
	S.	9.3	6.6		20

0.15' to 0.40') while the variation coefficient K decreases (from abt. 4 to 3 for threshold I and from abt. 4.5 to 3.5 for threshold II). Whether the variation coefficient in reality decreases cannot be settled on the basis of our present knowledge, but there is in any case no question of a diminishing of the percentage accuracy with the eccentricity. It is further seen that the variation coefficient is on the whole larger for threshold II than for threshold I.

When comparing the results from the range 5° to 15°

with the corresponding figures for the range 0° to 2° the reservation must be made that only one of the subjects has participated in both experimental series. The experiments with subjects A and S were carried out in the retino-nasal meridian, that with subject V in the retino-temporal. It appears from table VII and fig. 13b that the dispersion S increases with the eccentricity, and that the variation coefficient is constant (abt. 6 at threshold I). The only measurement which exists at an eccentricity of 15° is considerably higher, however, (abt. 10). For threshold II the figures are somewhat higher than for threshold I as in the range from 0° to 2°. The fact, that the percentage accuracy is lower in the range from 5° to 15° than in the more central area, is probably due to the circumstance that owing to the curtailment of the visual field, it was impossible to use an artificial pupil at eccentricities larger than 5°.

B. The Dependence of the minimum separabile on the Retinal Meridian

As mentioned on page 62 already *Aubert & Foerster* (1857) found that the rate at which the visual acuity measured by the min. separabile for objects illuminated from before decreased with increasing eccentricity was higher in the vertical meridian than in the horizontal. If points in the visual field having the same min. separabile are connected the result will be curves in the form of ellipses placed transversely. *Hirschberg* (1878) called these figures isopters, a name which is still used frequently. *Wertheim* (1894) found that for objects consisting of gratings illuminated from behind the isopters were roughly parallel with the outer boundaries of the visual field, so that the rate at which the visual acuity decreased was greatest in the upper part of the vertical meridian of the visual field, less high in the lower part of the meridian, still lower nasally, while the visual acuity was best temporally. No essential objections have since been put forward against these results.

As the technique of the experiments mentioned was not such as to render it possible to draw any conclusions with regard to the structure of the fovea (vide p. 62), it was desirable to perform new experiments on this problem as part of the present investigation. For this purpose the min. separabile was determined up to an eccentricity of 2° (in one experiment to 5°). The squares were 40" by 40", they were luminous on unilluminated ground; the line connecting the two squares was always horizontal, irrespective of the meridian examined. Readings were taken in two meridians during one experiment, in order to make the results more comparable, as the day-to-day variations (vide p. 104) were hereby eliminated.

By the experiments the min. separabile (vide table VIII) for 4 out of 5 subjects was found to be smaller in the retino-nasal or retino-temporal meridian than in the retino-superior from abt. 2° and outwards. For the fifth subject (M) the opposite was the case, although the difference found was so small that it could hardly be taken into account. No definite difference could be verified between the two half parts of the horizontal meridian, although the slight difference shown by the figures is of the same tendency as the one found by *Wertheim*.

The different positions of the isopters in the various meridians can be explained by the same theories which were applied to the variation of indirect vision along the individual meridians, namely, optical factors, practice and attention as well as structural factors. The difference in the refraction in the vertical and horizontal meridian plays hardly any role at eccentricities smaller than abt. 10° (vide p. 52), nor is it probable that practice effects or attention should be especially attached to the horizontal meridian. Among structural factors the distribution of the cones will, within an eccentricity of abt. 6°, scarcely be of any importance, but quite possibly more peripherally. By his countings *Østerberg* (1935[76]) thus found the same reduction in the number of cones per unit of area in all meridians within this limit, while peripherally the number

Table VIII.

The minimum separabile (threshold I) at various retinal meridians.

a. Retino-nasal and retino-superior meridians.

	0	30'	1°	1°30'	2°	Eccentricity
Subject G.	3.9'	4.6'	6.7'	8.7'	9.4'	retino-nasally
		5.3'	7.8'	10.6'	12.5'	retino-superiorly
Subject M.	3.5'	5.5'	6.4'	8.5'	10.1'	retino-nasally
		5.3'	6.4'	7.5'	9.7'	retino-superiorly
Subject S.	3.3'	5.0'	7.5'	9.6'	11.6'	retino-nasally
		5.4'	8.1'	11.0'	12.8'	retino-superiorly

b. Retino-nasal and retino-temporal meridians.

	0	30'	1°	1°30'	2°	Eccentricity
Subject G.	3.8'	5.7'	9.0'	10.9'	13.8'	retino-nasally
		5.5'	8.6'	10.6'	14.4'	retino-temporally
Subject M.	3.6'	5.5'	6.9'	7.6'	9.0'	retino-nasally
		5.4'	6.7'	8.6'	10.3'	retino-temporally
Subject S.	3.9'	5.3'	7.7'	10.1'	12.2'	retino-nasally
		5.3'	7.9'	10.5'	12.3'	retino-temporally

c. Retino-temporal and retino-superior meridians.

	0	30'	1°	1°30'	2°	Eccentricity
Subject A.	3.0'	4.1'	5.2'	6.4'	8.0'	retino-temporally
		4.5'	6.3'	8.1'	10.2'	retino-superiorly
Subject V.	3.5'	5.6'	7.0'	8.5'	9.5'	retino-temporally
		7.5'	8.2'	9.8'	12.0'	retino-superiorly

of cones in the retina decreased more rapidly downwards and temporally than upwards and nasally.

It is futher a probability that the functional units (»cone groups«) have oval shape with the longest diameter horizontally, and this would probably have the effect that the min. separabile for squares placed side by side would be larger than

Table IX.

The minimum separabile with the squares in different positions.

a. 0—2° retino-nasally. Average for 3 subjects (G.M.S.).

	Ecc. 0	30'	1°	1°30'	2°	Position of squares
Threshold I	4.1'	5.9'	7.9'	9.8'	12.1'	Side by side
	4.1'	5.5'	7.8'	8.3'	10.8'	On top of each other
Threshold II	3.2'	4.8'	6.4'	8.0'	10.0'	Side by side
	3.2'	4.5'	6.2'	7.6'	8.9'	On top of each other

b. 0—5° retino-temporally.

	Ecc. 0	2°	4°	5°	Position of squares
Threshold I	3.0'	5.2'	7.2'	8.8'	Side by side
	4.6'	5.5'	7.4'	8.3'	On top of each other
Subject A.					
Threshold II	1.7'	3.1'	3.9'	5.5'	Side by side
	2.9'	3.8'	5.3'	6.2'	On top of each other
Threshold I	3.6'	6.4'	8.5'	11.3'	Side by side
	7.5'	7.3'	8.4'	11.0'	On top of each other
Subject V.					
Threshold II	1.6'	3.3'	4.2'	5.8'	Side by side
	3.6'	5.0'	4.9'	6.7'	On top of each other

for squares placed on top of each other. In order to find out whether this is the case, some determinations of the min. separabile were carried out at eccentricities up to 5° and with squares placed either side by side or on top of each other. According to table IX, in which the results are recorded, the position of the squares seems to have no well-defined influence.

It is seen from table IX b that the central min. separabile for the two subjects A and V is smaller for squares side by side than for squares on top of each other. This must probably be taken to indicate a slight astigmatism in the two subjects. At eccentricities of 4° and 5°

no differences could be traced which seems to show that a slight astigmatism has a certain effect on central vision, but a much smaller one on indirect vision.

The difference in the size of the functional units which was found to exist at small eccentricities ($< 5^{\circ}$) must consequently probably be due to a corresponding difference in the distribution of nerve fibres to the individual retinal meridians.

C. The Influence of Attention and Fixation Target on the minimum separabile.

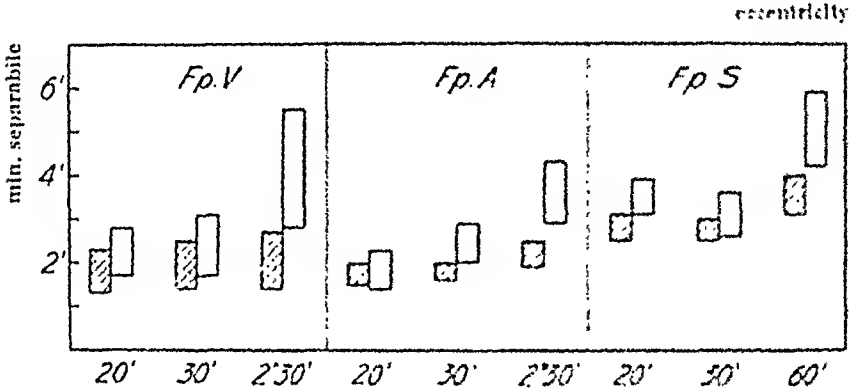
It has previously been mentioned (p. 54) that the attention seems naturally attached to central vision. This may e.g. be observed in connection with reading, when the peripheral visual field is so much relegated to the background of the mind that it hardly seems to exist. If it is therefore the intention to observe an object by indirect vision, it must be endeavoured to divide the attention between the central and indirect vision, i.e. fixating a certain point with central vision and at the same time focussing the attention as far as possible on the indirectly seen object. The fact that the acuity of central vision decreases hereby was observed by *Purkinje* (1825 [21]) and later by *Holm* (1923), but as no quantitative experiments has hitherto been carried out, it was thought desirable to perform new, orientating experiments on this problem.

One aim of these experiments was to determine the extent of the central visual field on which it is possible to fix the attention simultaneously without diminishing the acuity of central vision, another to find out whether indirect vision suffered any detrimental influence by the attention being especially concentrated on central vision. A number of experiments with various fixation targets were finally performed in order to determine whether the choice of fixation target was of any importance for indirect vision.

Two pairs of luminous squares, 50" by 50", on unilluminated ground were used for the experiments. One pair of

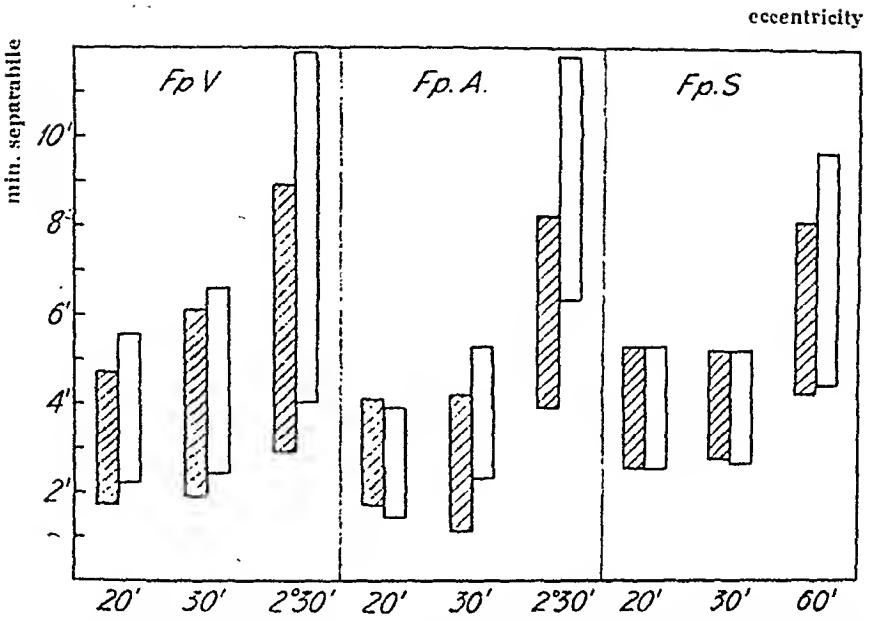
Fig. 14.

The minimum separabile for luminous squares on unilluminated ground, measured in the same experiment with two pairs of squares placed so as to be seen by central and indirect vision respectively, the attention in some of the series of readings being focussed on one set of squares, in others on both. The upper edge of the rectangles represent threshold I and the lower edge threshold II. The eccentricity of the squares seen indirectly is indicated by the numbers below the figure.
Ordinate: Min. separabile in min. of arc.



a. The min. separabile determined by *central* vision. For each of the three subjects readings have been taken with the indirectly seen squares placed at three different eccentricities. ⁒ represents the min. separabile for central vision when the attention is concentrated on the "fixation squares". □ denotes the same min. separabile when the attention during the readings includes the indirectly seen squares as well.

squares was used as fixation target, the other was placed at a certain eccentricity. For each subject an experiment was carried out at each of the following eccentricities: 20', 30', and 2°30' (or 1°), each experiment consisting of eight series of readings, and each series consisting of four individual readings of both thresholds. In the first series the min. separabile was determined centrally, in the second it was determined at the eccentricity in question — the subject being instructed to regard the other pair of squares as merely an ordinary fixation target and not to worry whether the squares appeared discrete or not. The »fixation squares« were fixedly adjusted according to the figure found for threshold I by the first series of readings. In the third series of readings the distance between the central squares was also the same. In this case the subject was instructed to make sure that he could at any time see the fixation squares separated, and the min. separabile was then determined by indirect vision. In the fourth series of readings the experiment was performed in the opposite manner, the



b. The min. separabile determined by *indirect* vision. The min. separabile is determined for 3 subjects at three different eccentricities. ▨ denotes the min. separabile by indirect vision, the squares seen by central vision being regarded as fixation target only. □ denotes the same min. separabile when it is endeavoured to divide the attention equally between both sets of squares.

squares seen indirectly being adjusted according to threshold I as determined in the second series. The subject now had to see these squares separated, while the min. separabile was determined centrally. The four last series of readings were the same as the four first, but in the reverse order, so that an average could be taken of each pair of series. The results of the experiments are recorded in fig. 14.

I can be seen from the graph that the central vision in all three subjects deteriorated (the min. separabile increased) when the attention could not be concentrated on central vision only, but had to include the indirectly seen object, even if this was placed at a distance of 20' only from the centre. When — conversely — the min. separabile was determined by indirect vision, and the attention had simultaneously to be directed against the fixation target, then the min. separabile increased as well, i.e. the indirect vision deteriorated. This, however, did only occur when the eccentricity was above a certain minimum, the size of the minimum differing for the three

different subjects. In subject S. f. inst. the min. separabile did not increase by divided attention at eccentricities of 20' and 30', but at 60', while subject A was able to cover an eccentricity of 20' but not 30', before the min. separabile increased. In subject V the indirect vision deteriorated already at an eccentricity of 20' when the attention was divided.

It can thus be concluded from the experiments that it is hardly possible to encompass a central area of the visual field larger than a part of the fovea with the same amount of attention. The experiments moreover confirm the observation made by *Purkinje* that if the attention is diverted from the central to the indirect vision, or vice versa, the visual acuity is reduced in the central or peripheral area respectively.

The explanation of the fact that the physiological resolving power can thus be altered by a central factor is probably to be found in a »displacement of the thresholds« by the subject under the existing conditions (conf. p. 104).

In conjunction with the investigations on the influence of attention on indirect vision an experiment on the suitability of various figures as fixation targets shall be mentioned here. It would be quite reasonable to assume that fixation targets of different shape did not attract the attention equally much, or that it was more difficult to fixate e. g. a point object than a cross-hair continuously for some time, as the latter would stimulate a larger retinal area and would consequently hardly be so susceptible to fatigue-phenomena. A defective fixation would probably manifest itself in a tendency of the fixation axis to deviate in the direction of the indirectly seen object, and especially so by small eccentricities. On account of these deviations it would be natural to assume that paracentrally the min. separabile was found to be smaller for an inferior than for a good fixation target.

In order to investigate this problem the min. separabile was determined at an eccentricity of 30' with four different fixation targets. The series of readings were each carried out twice and in different order with the three subjects.

The four fixation targets were:

a. A luminous square, 40" by 40", on unilluminated ground.

b. A luminous square, 4' by 4', on unilluminated ground.

c. An unilluminated square, 4' by 4', on luminous ground.

d. A luminous T-shaped slit, each of the limbs being 15' long measured from the point of intersection. (The object was of somewhat larger dimensions than the fixation target usually applied (conf. p. 87).

The test object consisted of two luminous squares, 40" by 40", on unilluminated ground. The luminous intensity of the fixation target or its background was kept constant somewhat above threshold brightness. As result of the experiments, vide table X, no marked difference in the values corresponding to the four fixation targets was found. The experiment thus did not furnish any proof that the shape of the fixation target has an influence on the min. separabile by indirect vision.

Table X.

The minimum separabile at an eccentricity of 30' by different fixation targets.

(Average values for 3 subjects.)

Fixation target:	Luminous square 40" by 40"	Luminous square 4' by 4'	Unilluminated square 4' by 4'	Luminous T-shaped slit
Threshold I	5.0	4.9	5.0	1.5
Threshold II	2.1	2.2	2.1	2.4

D. The Dependence of the minimum separabile on Illumination.

1. The Influence of Intensity.

The authors who have investigated the variation of the min. separabile for luminous objects with the illumination of the object have obtained different results (vide pp. 28 and 29). While *Berger & Buchthal* (1938 a) states that the min. separabile increases uniformly with increasing illumination and

increases most for small squares, less for large squares, *Hecht & Mintz* (1939) maintains that the opposite holds in the case of very large objects. And it was finally found by *Wilcox*

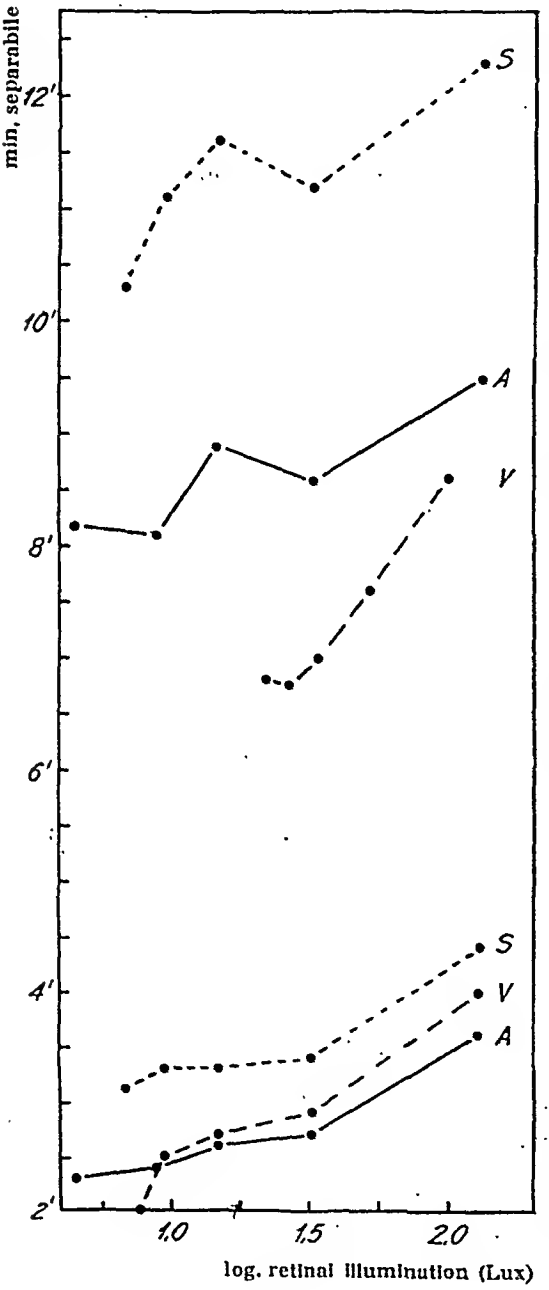
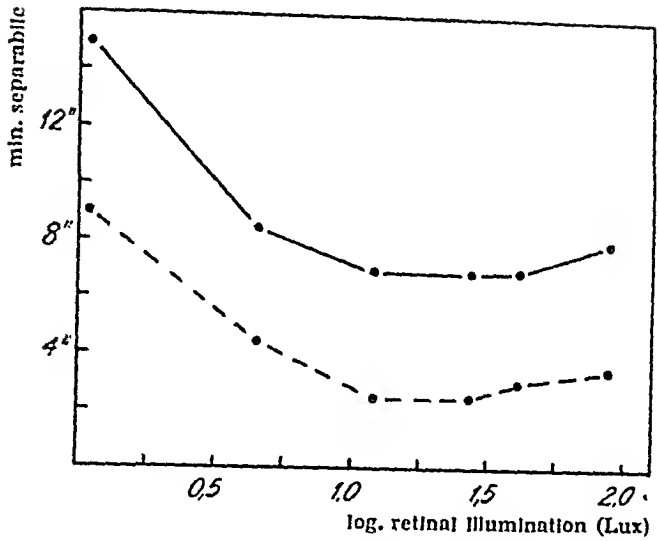


Fig. 15.

The minimum separabile at a *retinal illumination varying from threshold brightness to glare.*
Objects: luminous squares on unilluminated ground.
Abscissa: the logarithm of the retinal illumination in lux.
Ordinate: min. separabile in min. of arc or sec. of arc.

a. Experiments with *small squares*, 40″ by 40″. To render the graph more perspicuous only threshold I has been plotted. The experiments were carried out centrally and by indirect vision with 3 subjects.



b.

b. Experiments with *large squares*, 13' 20" by 13' 20". by central vision. Average for 2 subjects (A. and V.).

(1932) that for objects of medium size the min. separabile first decreases to a certain minimum and then again increases.

As it has previously (p. 31) been pointed out neither *Wilcox'* nor *Hecht & Mintz'* results are directly comparable with those of *Berger & Buchthal,* as the latter are the only investigators who have kept the eyes of the subjects properly and constantly light-adapted. It is not certain, however, whether this fact can explain the difference between the experimental results. Perhaps the reason is to be found in the highly diverging sizes of the objects. The fact that the curve found by *Wilcox* (with objects of medium size) can be regarded as lying between the results found by *Berger & Buchthal* and those of *Hecht & Mintz* speaks in favour of this theory.

In explanation of their results *Berger & Buchthal* say that by increasing retinal illumination the dispersion of the light in the eye tend to shift the border lines between illuminated and unilluminated retinal areas at the cost of the unilluminated areas, i. e. in this case the space between the squares. For larger objects, as those used by the other authors, the min. separabile is smaller than the optical resolving power of the eye. The »unilluminated« space between the images of the object will

in this case never be quite unilluminated. As a consequence of this the min. separabile is a function of the differential threshold for this particular area, i. e. quite a different function from the one investigated by *Berger & Buchthal* (conf. p. 31).

In order to test the tenability of these view points and — what has not been done before — to verify *Berger & Buchthal's* results, experiments were carried out on the min. separabile with squares of varying brightness. The experiments were performed with squares of two different sizes, one 40" by 40" and another 13'20" by 13'20". The latter squares were twice as large as the largest squares used by *Berger & Buchthal,* but the length of the side was only ⅔ of the length of the luminous slits used by *Wilcox.* As the arrangements with the wedges could not be used for such large squares, they had to be prepared as one contiguous rectangle divided in halves by copper wires of varying thickness (vide p. 93). In addition to the experiments performed centrally others were carried out by indirect vision (at an eccentricity of 2° or 5°), as no records of such experiments seem to exist.

The experimental results appear from fig. 15. In conformity with the results found by *Berger & Buchthal* (1938 a) it was possible with small squares to trace a uniform increase of the min. separabile with increasing retinal illumination from threshold brightness to glare.

By indirect vision the results were the same, but the shape of the curve was more irregular. In the case of the large squares (fig. 15) the course of the curves is different from that corresponding to the small ones. The min. separabile starts by diminishing with increasing retinal illumination, then it remains constant for some distance and finally it increases slowly. The shape of the curve thus corresponds to the one described by *Wilcox* and to the dependence of the differential threshold on the illumination (vide p. 7).

The experiments on the dependence of the min. separabile on the illumination thus confirm the hypothesis from which we started, namely that the result depends upon the size of the squares used. In the case of small lumi-

nous squares, where the min. separabile is a measure of the size of the functional units, it increases uniformly with the retinal illumination both by central and indirect vision. In the case of luminous squares which are so large that the min. separabile determined is a function of the intensity discrimination of the eye, it varies with the intensity in conformity with this fact.

2. Continuous Illumination and Momentary Illumination.

The only experiments on the dependence of the min. separabile on the exposure time of the object, which are recorded, were made by *Graham & Cook* (1937) with a luminous grating as test object. According to these experiments the min. separabile decreases with increasing illumination time up to a certain point, but as the experiments are performed during slight dark adaptation and with such a large object that the min. separabile is a function of the intensity discrimination, it was found desirable to carry out new experiments.

The min. separabile was therefore determined for luminous squares (40" by 40"), with series of threshold determinations, both by continuous illumination and momentary illumination of varying duration. The experiments were carried out both centrally and at an eccentricity of 2° retino-nasally. The momentary illumination of the squares was produced by placing a photographic shutter (vide p. 89) before them. The experimental results are seen from fig. 16. While the min. separabile by central vision seems to be independent of the exposure time, threshold I at an eccentricity of 2° is considerably lower for momentary illumination than for continuous, and the difference is the same whether the time of illumination is 0.07 or 4 seconds. The influence on threshold II is not so certain, however. The result by central vision was verified by an experiment with a great number of threshold determinations (40 readings, divided in 8 series on two days) both by continuous illumination and by an illumination of 1 second.

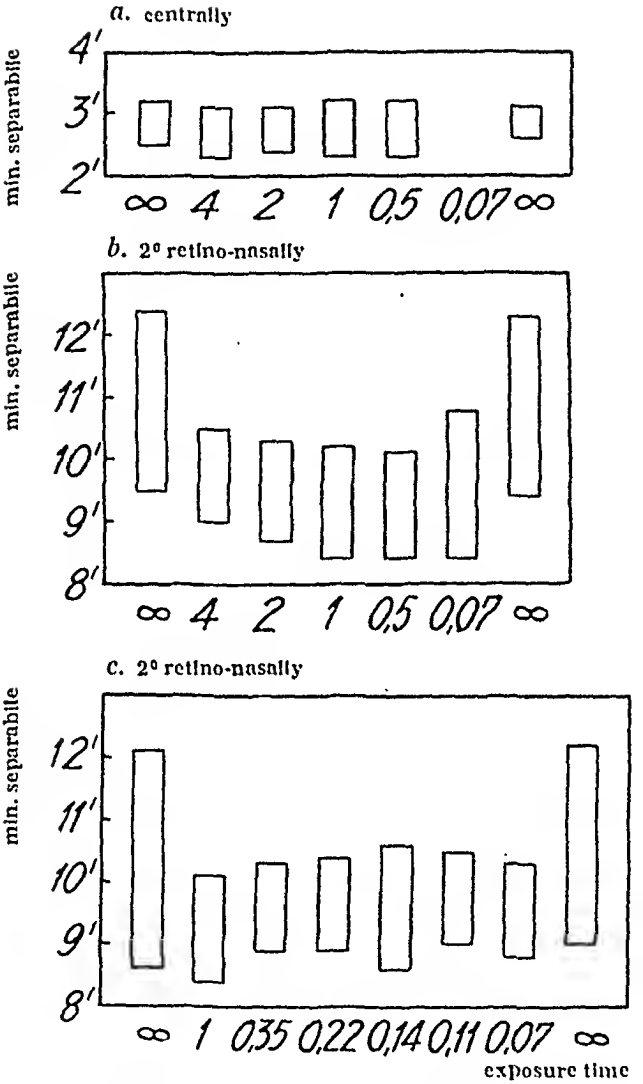


Fig. 16.

Comparative experiments on the min. separabile by *continuous illumination* and by *momentary illumination*.
Objects: Luminous squares, 40″ by 40″, on unilluminated ground. Average for 3 subjects (M, G, S).
Ordinate: Min. separabile in min. of arc. The upper edge of the rectangle represents threshold I, the lower edge threshold II.
a. Central vision. The min. separabile by continuous illumination (∞) and momentary illumination from 0.07 to 4 seconds.
b. At an eccentricity of 2° retino-nasally. The same exposure time as centrally.
c. Same experiment by continuous illumination and momentary illumination from 0.07 to 1 second.

Vide table XI. In addition to the min. separabile the table records the dispersion of the threshold determinations calculated according to the formula mentioned on page 111).

Table XI.

The minimum separabile by central vision for luminous squares on unilluminated ground by continuous illumination and by illumination periods of 1 second.

Average for 3 subjects (M. G. S.). Each figure is the result of 120 readings.

	Continuous Illumination:		*Momentary Ill. of 1 second:*	
	Minimum separabile	Dispersion of threshold determinations	Minimum separabile	Dispersion of threshold determinations
Threshold I:	204"	13"	186"	12"
Threshold II:	164"	11"	152"	12"

It might be assumed that the influence of the exposure time was dependent on the retinal eccentricity. In order to investigate this, the min. separabile was determined at various eccentricities and with momentary illumination (0.11 sec.), vide fig. 17. It appears from the curve that the difference between the two thresholds is smaller by momentary illumination than by continuous illumination, so that the divergence with increasing eccentricity is only slight. This seems mainly to be due to a diminishing of threshold I, a fact which may be explained in several ways.

Broca & Sulzer (1902) and *Lehmann* (1905 [183]) have observed that the brightness of sensation is increased by momentary illumination, probably the same phenomenon as the one that manifests itself electro-physiologically in an increase in the impulse frequency (*Adrian & Matthews,* 1927 a). It is not very probable that the phenomenon has any influence on the min. separabile when the latter is a measure of the size of the functional units. Besides it is hardly probable that such an effect would occur by a »momentary illumination« lasting 4 seconds. The high figures for the min. separabile by continuous illumination might also be due to an adaptation to the stimulus. (Adaptation is here taken to mean the state of habituation towards a stimulus observed in all sensory organs. As an example it may be mentioned that the receptors of the skin quickly adapt themselves

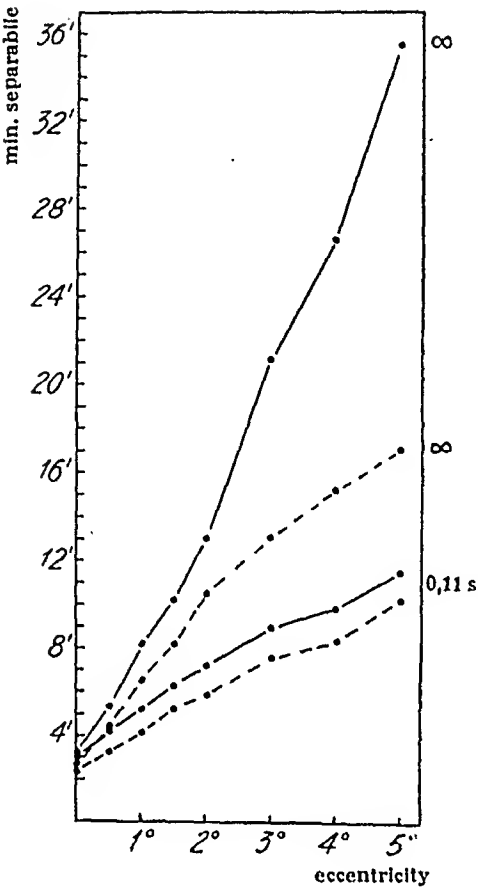


Fig. 17.

The minimum separabile at *various retinal eccentricities by continuous illumination (∞) and momentary illumination* (0.11 sec.).
Object: Luminous squares, 40″ by 40″, on unilluminated ground. Average for 3 subjects (M, G, S).
Abscissa: Eccentricity in degrees.
Ordinate: Min. separabile in min. of arc.
——— Threshold I.
– – – – Threshold II.

to constant surroundings so that the sensation vanishes completely. Whether the phenomenon should be called fatigue or adaptation is mostly a matter of definition (*Adrian* 1928 [106]). It can be seen from the following that the adaptation may have a different influence on the two thresholds. While by determination of threshold II the retinal images of the two squares converge towards each other on receptors not stimulated on beforehand (»not fatigued«), the opposite takes place when threshold I is being determined. Here it is a question of perceiving the space between the squares, but as these recede from each other the increase of the space falls on receptors which the image of the squares has just left — i. e. the receptors are in a stimulated (»fatigued«) state. This phenomenon will cause an increase of threshold I only and will disappear by momen-

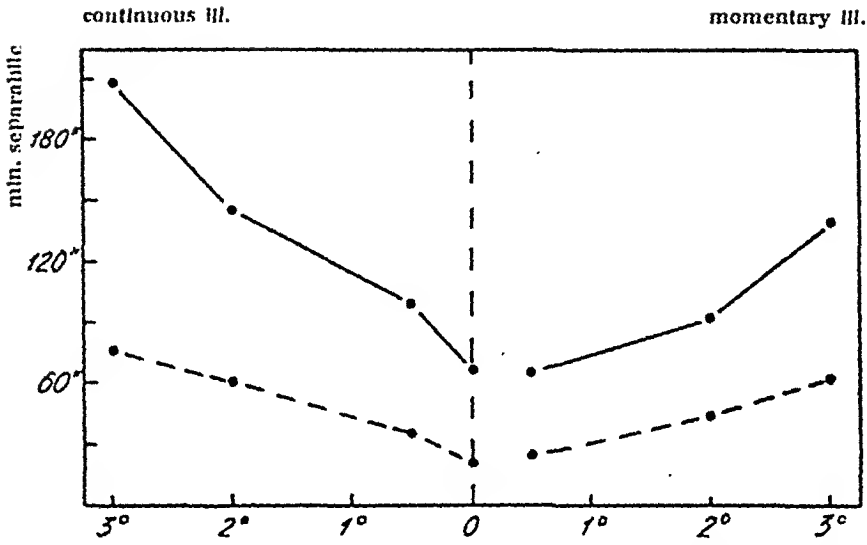


Fig. 18.

The minimum separabile for *unilluminated squares* (3' 20" by 3' 20°), *on luminous ground at various retinal eccentricities, by continuous illumination and momentary illumination (0.07 sec.).* Average for 2 subjects (A and V).
Abscissa: Eccentricity in degrees.
Ordinate: Minimum separabile in min. of arc.
– – – – – Threshold I.
——— Threshold II.

tary illumination. The argumentation is based on the assumption that the retinal images fall on a very limited number of receptors, and this is probably a fact (vide p. 58). If the images kept on moving over a considerable number of receptors they would hardly be able to exhaust all the stimulated receptors.

If the influence of the momentary illumination were exclusively due to adaptation, then it might be expected that momentary illumination in the case of unilluminated squares on luminous ground would cause an increase of threshold II. The result of such experiments were, however, another (vide fig. 18). The distance between the two thresholds diminished by momentary illumination — just as for luminous squares — but both thresholds diminished, threshold I more than threshold II. Consequently it will not be possible to explain the effect of momentary illumination by the theory advanced here.

Besides by adaptation, it would also be possible to explain the experimental results obtained by momentary illumination by assuming an alternating activity of the retinal

functional units. Elsewhere in this book (pp. 34, 156 and 179) an account has been given of the view point that it is hardly possible to explain neither the dependence of the form sense on the illumination nor *Troxler's* phenomenon in any other way.

If the functional units, when subjected to constant stimulation (continuous illumination), are imagined to keep on alternating between an active and a relatively refractory period, it is natural to assume that the units, when unilluminated, are constantly in a state of responsiveness. When the units are stimulated by momentary illumination a larger number of units will be active per unit of area, the retinal mosaic is consequently finer and the min. separabile will decrease. As it is the units corresponding to the space between the squares which determine the size of the min. separabile, the alternating activity will only manifest itself by unilluminated squares on luminous ground.

In addition to the »fatigue theory« mentioned on page 130 the assumption of an alternating activity of the functional units will furnish an explanation of the values for the min. separabile found by momentary illumination.

E. The Dependence of the minimum separabile on the Size of the Squares.

Hofmann (1925 [34]), *Guillery* (1931 [763]) and other, both earlier and later, authors assume that the min. separabile by central vision is under no experimental conditions independent of the size of the object, but varies always with the latter (vide p. 22). Consequently it should not be possible to find any value of the min. separabile which might be considered to be a measure of the fineness of the retinal mosaic and thereby constitute a natural unit for the measurement of visual acuity.

In contradistinction to this *Berger & Buchthal* (1938 a) have found (vide p. 23) that the min. separabile for luminous squares of threshold brightness on unilluminated ground is constant for squares the retinal image of which is less than 18 sq. μ, but diminishes when the image is larger. If, however, the back-

ground is not unilluminated, but illuminated from before, then the min. separabile decreases uniformly with increasing size of the squares, probably because in this case the min. separabile is a function of the intensity discrimination. When finally the min. separabile is determined for unilluminated squares on luminous ground, it is observed to be constant for retinal images convering more than abt. 650 sq. μ (*Berger* 1939).

The explanation of the constant values of the min. separabile for luminous quares is, that in this case the min. separabile is a function of the a v e r a g e d i a m e t e r of the functional units, in the case of unilluminated squares it is a measure of the size of the s m a l l e s t functional units.

As it thus seems to be possible to draw conclusions with regard to the dimensions of the functional units from the min. separabile, it was found desirable to verify *Berger & Buchthal's* and *Berger's* results by central vision. Corresponding experiments by indirect vision were also carried out in order to determine the maximum retinal eccentricities at which separated functional units are to be found (conf. p. 68). The objects used for the experiments were, in addition to luminous squares on unilluminated ground, unilluminated squares on luminous ground and the same objects illuminated from before.

In order to render the representation more perspicuous the experimental results for each object will be dealt with separately, account being given first of the experiments by central vision then by indirect vision.

1. Luminous Squares on Unilluminated Ground.

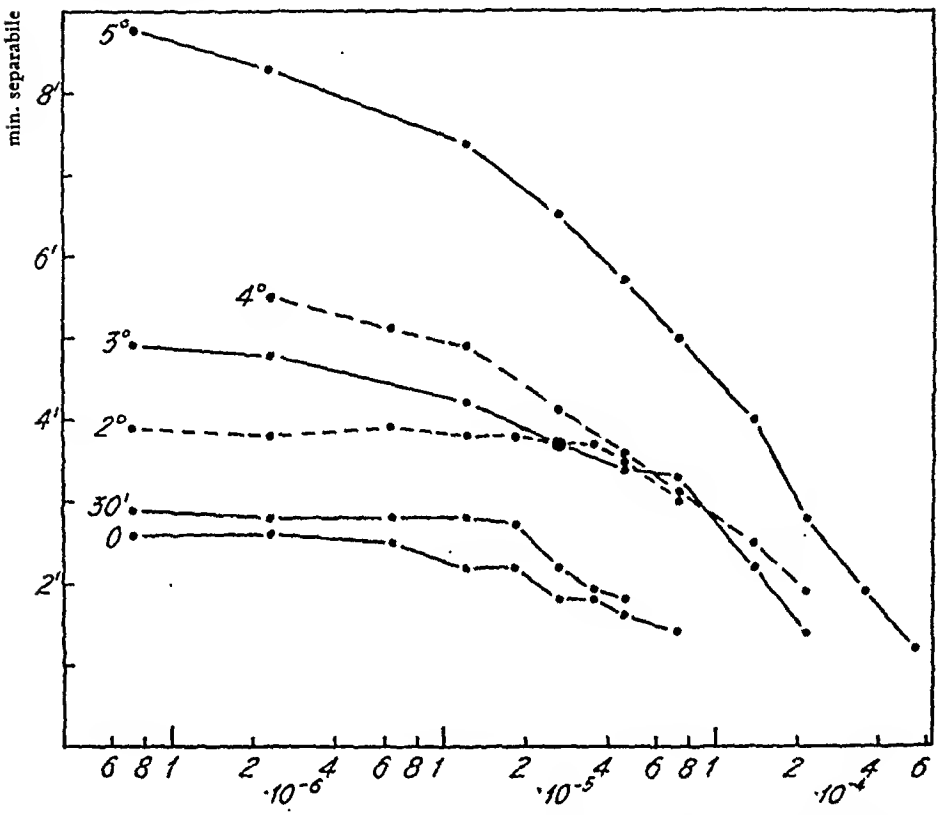
a. C e n t r a l v i s i o n.

For luminous squares on unilluminated ground and by central vision (fig. 19) the same dependence on the size of the square as the one described by *Berger & Buchthal* (1938 a) was found, the min. separabile being constant for squares the length of side of which is smaller than 30" to 40" (2.5μ to 3.2μ) corresponding to a retinal image of 6 to 11 sq. μ. For still larger squares the min. separabile increased uniformly with increasing size of square within the range investigated.

The course of the curve can hardly be explained in any

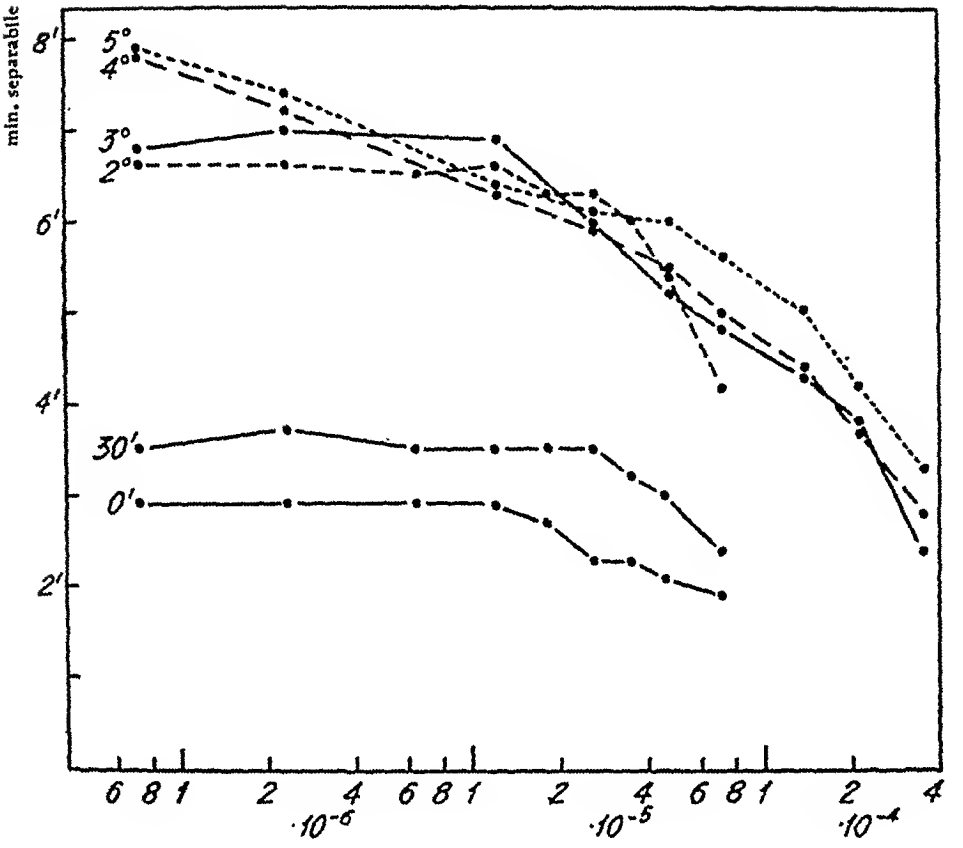
Fig. 19.

The dependence of the minimum separabile on the size of the square by *luminous squares of threshold brightness on unilluminated ground.* To make the graph more perspicuous only threshold I has been plotted.
Abscissa: The area of the retinal image of the square in sq. mm (Log. scale).
Ordinate: The min. separabile in min. of arc.



a. Experiments with subject A, centrally and at eccentricities of 30', 2°, 3°, 4° and 5° retino-nasally.

other way than the one applied by *Berger & Buchthal* (1938 a). The constant value of the min. separabile for small squares corresponds to the average diameter of the functional units in the area of the centre of the fovea across which the image travels on account of the involuntary oscillating movements of the eye. *Berger & Buchthal* assume that the size of the individual functional units varies somewhat. The break in the curve is explained in the following way: when the images of the squares become larger than the smallest functional units, then the statistical chance that one of the latter is enclosed

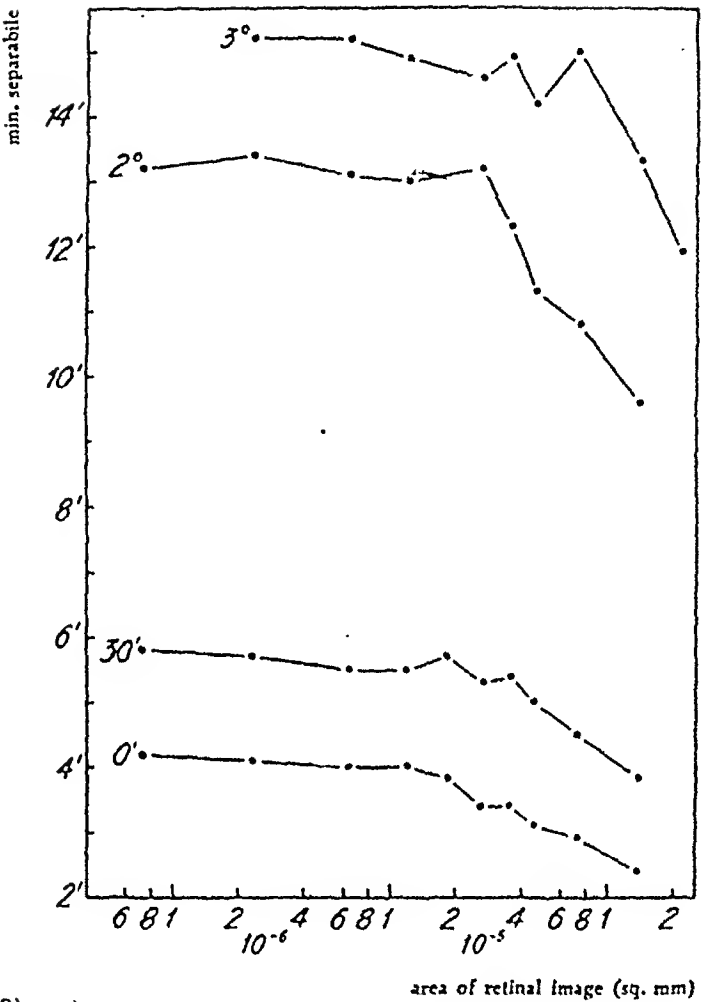


(Fig. 19)

b. Experiments with subject V, centrally and at eccentricities of 30′, 2°, 3°, 4° and 5°, retino-nasally.

between two images will increase. As the min. separabile may be regarded as the average value of a series of observations it will decrease correspondingly.

The explanation of the course of the curve become slightly more perspicuous when the size of the diffraction disc (vide p. 15) is taken into consideration. If the diffraction disc is assumed to be smaller than the smallest functional units, the min. separabile will not decrease until the retinal image becomes larger than the units. If, on the contrary, the diffraction disc is larger than the functional units, it will not be possible for the retinal image to grow, and the min. separabile will not change until the size of the image of the square exceeds that of the diffraction disc. As far as it is known, both



(Fig. 19)

c. Experiments with subject S, centrally and at eccentricities of 30', 2° and 3°, retino-nasally.

the diffraction disc and the smallest functional units (the cones) have a size of abt. 1 min. of arc (vide pp. 16 and 18). It must therefore be a justified assumption that the constant values of the min. separabile for small squares are due to the fact that the luminous squares, when adjusted at their proper threshold brightness, are reproduced on the retina as diffraction discs. The illumination of the latter depends on two factors, the light intensity and the size of the squares. When the size is increased, a point will be reached, when the size of the image will also increase, even if the retinal illumina-

tion is reduced, so that the image can no longer be said to be a diffraction disc. If the image becomes larger than the smallest functional units the min. separabile will decrease and the before mentioned break in the curve will occur.

If the circumstance that one of the smallest functional units is enclosed between the images of the squares should

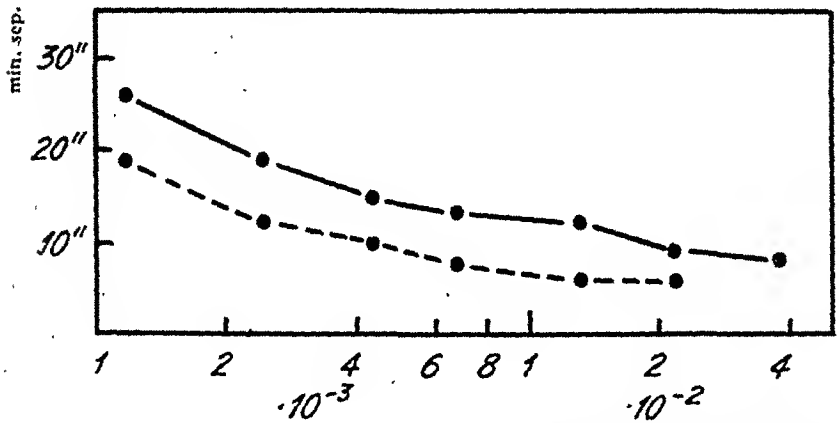


Fig. 20. The dependence of the minimum separabile on the size of the square for *very large luminous squares of threshold brightness on unilluminated ground.* Average of experiments with two subjects (A and V), *centrally.*
Abscissa: Area of the retinal image of a square in sq. mm (Log. scale).
Ordinate: Min. separabile in min. of arc.
——— Threshold I.
– – – – Threshold II.

make it possible to see the squares separately, then it must be assumed that the squares are seen to be contiguous where the space between them falls on larger units. This has also been described by *Berger & Buchthal* (1938 a), and it has been possible to confirm it by the present experiments.

Whether the min. separabile for luminous squares, in case of large squares, can reach a constant minimum value corresponding to the diameter of a cone, an optimum, as assumed by *Berger & Buchthal* (1938 a) and *Berger* (1939), does not appear from any of the experimental results given in these papers, as the experimental methods applied did not allow for sufficiently large squares. It has therefore been endeavoured to elucidate this problem through experiments with squares varying from 6'40" by 6'40" to 40' by 40'. The intermediate

space was formed by copper wires of varying thickness (for further details vide p. 92). It appears from fig. 20, on which the results have been plotted, that the min. separabile, within the range examined, decreases with increasing size of square to a value of 8", and thus does not reach any minimum value. The explanation must be found in the fact that the min. separabile has become so small that the dispersion of the light in the media and the diffraction fringe around the squares illuminate the space between them, so that the min. separabile becomes a function of the intensity discrimination of a row of units. (conf. p. 22).

b. Indirect vision.

According to fig. 19 the dependence of the min. separabile on the size of the object by indirect vision is not the same at the various eccentricities examined. At an eccentricity of 30', 2° and 3°, retino-nasally, the main parts of the curve are the same as by central vision, a horizontal part followed by a smooth decline. The level of the horizontal part is higher for larger eccentricities, and the length of it is larger at 30' than centrally, and it is even longer at 2°, but at an eccentricity of 3° it is quite short. Even more peripherally, at eccentricities of 4° and 5°, the horizontal part does not exist any longer, the min. separabile decreases uniformly, more and more, however, with increasing size of square.

The course of the curves for the eccentricities of 30' and 2°, retino-nasally, is quite naturally explained in the same way as the corresponding curve for central vision. The higher level of the horizontal parts — compared with the curves for central vision — is probably due to the fact that the mean diameter of the functional units increases with the eccentricity. While the functional units in the fovea must consist of a group of closely spaced cones, coupled to the same optic nerve fibre, the units outside the rod-free area must be composed of a number of cones more or less separated by interposed rods and must thus cover a larger area. When the retinal image of the square exceeds the size of the smallest

functional units at that particular eccentricity the min. separabile begins to decrease, just as it does centrally.

With its short horizontal part the curve for the eccentricity of 3° has such a shape that it seems to be a transition form between the corresponding curves for 2° and 4°, and this perhaps indicates the incipient influence of a new factor.

At an eccentricity of 4° and 5° the min. separabile decreases, in the beginning slowly and later on rapidly, with increasing size of object. The reason for this is probably that outside an eccentricity of 3° the functional units are no longer functionally separated and thus cannot be said to be units. It must be assumed that here the nerve impulses have a tendency to spread through the horizontal neural connections of the retina, which is consistent both with the anatomical structure of the retina (vide p. 71) and with results obtained by electro-physiological experiments as well as investigations on the fusion frequency of flicker (vide p. 76). When the nerve impulses disperse through the retina they will also spread to the otherwise unstimulated area between the squares, and the intensity threshold of the functional units here will decrease. Then min. separabile will consequently no longer be a measure of the retinal structure only, but also a function of the intensity discrimination. It can be seen that at an eccentricity of 4° and 5° there are two reasons, why the intensity discrimination is not the only factor determining the dependence of the min. separabile on the size of square. In the first place the min. separabile does not decrease quite uniformly with increasing size of object, but increases slowly at first and later on more rapidly. When, however, the object consists in squares illuminated from before, the min. separabile of which is a direct function of the intensity discrimination, the dependence is approximately linear (vide figs. 1 and 23). In the second place the min. separabile at an eccentricity of 5° increases with the retinal illumination (vide fig. 15 a p. 124) which the intensity discrimination does not (vide p. 7).

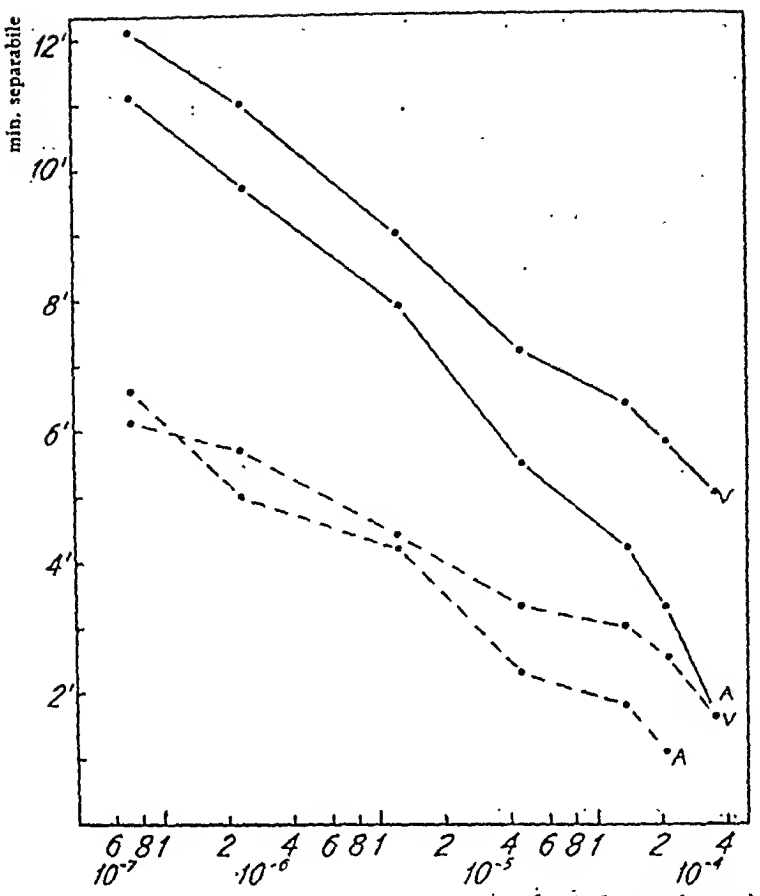


Fig. 21.

The dependence of the minimum separabile on the size of the squares in case of *luminous squares of threshold brightness on unilluminated ground*. Experiments with subjects A and V at an *eccentricity of 10°* retino-nasally.
Abscissa: The area of the retinal image of the square in sq. mm. (Log. scale).
Ordinate: Min. separabile in min. of arc.
——— Threshold I.
– – – – Threshold II.

An experiment with the two subjects A and V at an eccentricity of 10° (retino-nasally) (fig. 21) seems, however, to show that in this case the min. separabile is essentially a function of the intensity discrimination, as the curve is linear.

According to the preceding the retinal area, within which the min. separabile for luminous points of threshold brightness on unilluminated ground is a measure of the diameter of the functional units, must extend from the centre to an eccentricity of about 3° horizontally. In order also to determine the extent of the area vertically, experiments were carried out

in the retino-superior meridian. As the curve for an eccentricity of 2° had the same shape as the one for 3° retino-nasally, it may be concluded from the experiments, that separated functional units are only found in the retina up to an eccentricity of 3° horizontally and 2° vertically.

2. Unilluminated Squares on Luminous Ground.

a. Central vision.

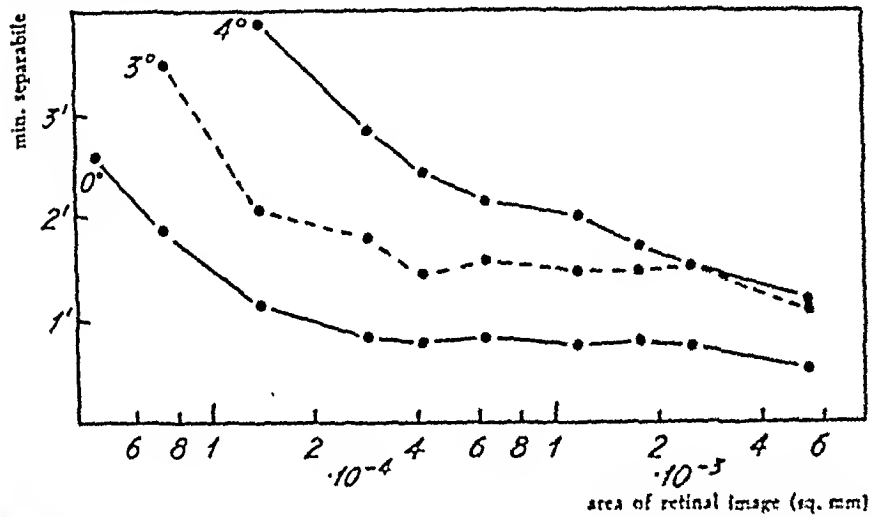
The dependence of the min. separabile on the size of squares was found by unilluminated squares on luminous ground of threshold brightness to be as shown on fig. 22.

By central vision the smallest squares still visible were 80" by 80". When the size of the squares increased the min. separabile decreased until it reached abt. 50", after which it kept constant, so that the min. separabile was independent of the size of the object. The result of the experiment was consequently the same as the one found by *Berger* (1939) with the same object, and the course of the curve can easily be explained as done by *Berger*. *Berger* assumes that the diameter of the individual functional units varies somewhat. When the size of the squares increases, then the statistical chance of one of the smallest functional units being enclosed between the sides of the squares also increases, until the squares have reached such a size that they, at any time, enclose a sufficient number of units to make it possible to recognize the squares as separate. When this point has been reached, a further increase of the size of the squares will have no influence on the min. separabile, which will remain constant, and the value of which (abt. 50") will correspond to the diameter of the smallest functional units.

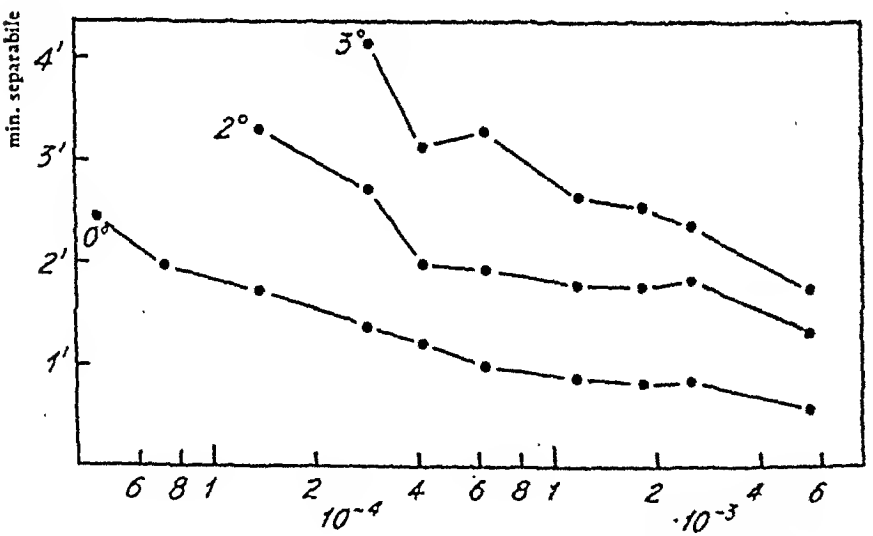
According to the experiments with luminous squares on unilluminated ground (p. 133) the diameter of the smallest units should be 30" to 40", while according to the experiments with unilluminated squares just mentioned they should be 50" wide.It will be understood that this difference is only apparent, when considering that the image of a luminous square on account of the dispersion of light in the eye will stimulate

Fig. 22.

The dependence of the min. separabile on the size of the square by *unilluminated squares on luminous ground of threshold brightness.* To render the graph more perspicuous only threshold I has been plotted.
Abscissa: The area of the retinal image of the square in sq. mm (Log. scale).
Ordinate: Min. separabile in min. of arc.

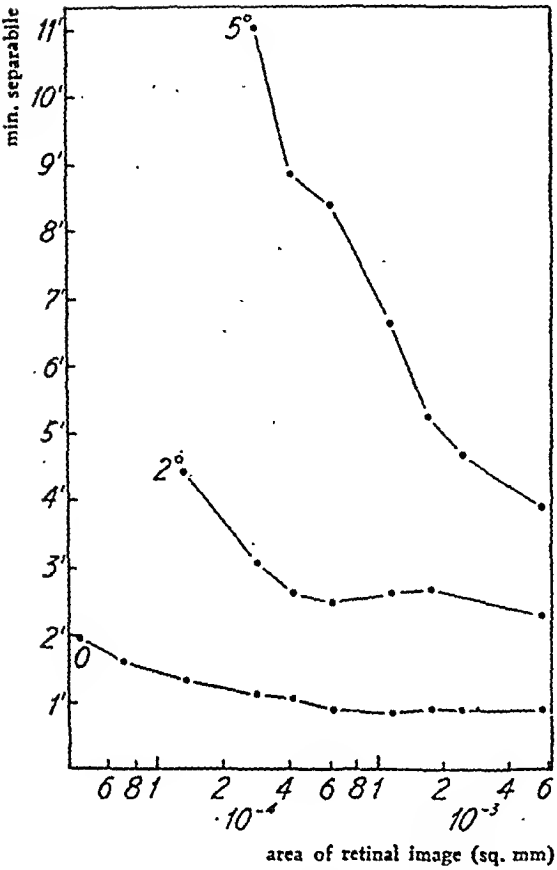


a. Experiments with subject A centrally and at eccentricities of 3° and 4° retinonasally.



b. Experiments with subject V centrally and at eccentricities of 2° and 3° retinonasally.

a larger area than the one corresponding to the geometrical image, while the opposite is the case for unilluminated squares. An unilluminated square will consequently be perceived as



(Fig. 22.)
c. Experiments with subject S centrally and at eccentricities of 2° and 5° retino-nasally.

smaller than a luminous one. If it is assumed that the dispersion of light shifts the boundary between illuminated and unilluminated area equally much in the two cases, then the diameter of the smallest functional units in the foveal centre should be 40" to 45" or 3.2 μ to 3.7 μ according to the experiments. This corresponds to the magnitude of the central cones in the fovea (vide p. 17) and as each of these cones constitutes a functional unit they must be connected to the optic centres of the cerebral cortex each by an isolated pathway.

b. Indirect vision.

As it was the case for luminous squares the curves for unilluminated squares were found to be at a higher level at eccen-

tricities of 2° (or 3°) than centrally (vide fig. 22), but they were of the same shape. At larger eccentricities (4° or 5°) the curve became more »straightened out«, the horizontal part being substituted by a smooth decline, so that every increase of the size of the squares resulted in a reduction of the min. separabile.

It is natural, as far as possible, to apply the same explanation to the course of the curves as the one used for the luminous squares. That the horizontal part of the curves is higher at an eccentricity of 2° than centrally is probably due to the fact that the smallest functional units are larger by indirect than by central vision, which was known on beforehand from histological investigations (vide p. 53). The »straightening out« of the curves at eccentricities larger than 3° may be explained by the absence of separated functional units here. The experiments with unilluminated squares thus further confirm this assumption, which has already been mentioned as a result of the experiments with luminous squares.

3. The minimum separabile for Twin-objects Illuminated from before.

All previous experiments on the dependence of the central min. separabile on the size of the object have given the same result when the object applied was white squares illuminated from before on black ground or black squares on white ground. Thus it was found first by *Aubert* (1865 [228]) and later on e. g. by *Hofmann* (1925 [32]) (vide fig. 1) and *Berger* (1936) that the min. separabile decreases uniformly with increasing size of square.

As it has been mentioned several times previously the min. separabile for luminous squares on unilluminated ground is, within certain limits, independent of the size of the object. If the unilluminated ground is illuminated slightly from the front, then the dependence according to experiments by *Berger & Buchthal* (1938 a) is the same as by objects illuminated solely from before. This result, which proved reproducible, (vide fig. 23) is according to *Berger & Buchthal* due to the influence of a special factor, probably the intensity discrimination of

light. The rôle played by the intensity discrimination by illumination from before is not further explained in the said paper. The most natural assumption would be that the illumination from before lights up the otherwise unilluminated space between the squares, so that the difference in brightness between squares and background is no longer unilluminated/luminous, but illuminated/more illuminated. Consequently the min. separabile will no longer be a function of the diameter of the unstimulated functional units, but a determination of the just perceptible difference between the illumination of the squares and of the intermediate space, i. e. a determination of the differential threshold, by which the area, and not the intensity, is varied (conf. p. 8). If this assumption is correct, then the usual break in the curve would also become levelled out for unilluminated squares on luminous ground in case the squares were illuminated from before. As it should be decisive for the shape of the curve whether retinal images of the dark squares were under the intensity threshold or not, it should also be possible for the dispersion of the light in the eye at high ground-intensity to spread the light over the images of the squares so much, that the min. separabile became a function of the intensity discrimination.

In order to verify these consequences of the theory about the importance of the intensity discrimination for the min. separabile two series of experiments were carried out. In one the object was two black squares illuminated from before and on luminous ground of threshold brightness. The illumination came from a lamp housing screened in the direction of the subject and open towards the object (vide p. 89). The housing contained a 38-w electric lamp and was placed at a distance of 1 m from the squares. The min. separabile was determined with squares of different sizes by central vision.

In the second series of experiments the min. separabile was determined for the same squares on luminous ground of high intensity without any illumination from before.

In both cases it was possible to record a uniform decrease of the min. separabile with increasing size of object (vide figs.

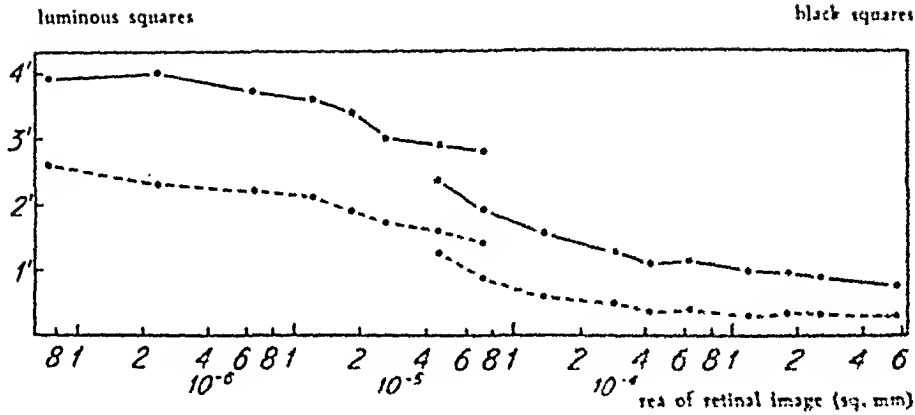


Fig. 23.

The dependence of the minimum separabile on the size of the square by *luminous squares of threshold brightness on black ground illuminated from before, and black squares illuminated from before on luminous ground of threshold brightness.* Average of experiments with 3 subjects (A, V and S) by *central vision.*
Abscissa: The area of the retinal image of the square in sq. mm (Log. scale).
Ordinate: Min. separabile in min. of arc.
——— Threshold I.
– – – – Threshold II.

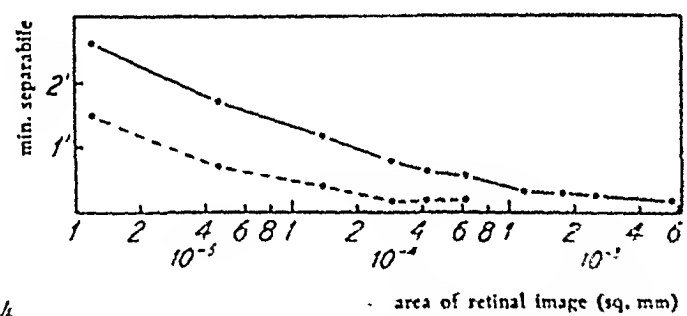


Fig. 24.

The dependence of the min. separabile on the size of the squares *by unilluminated squares on luminous ground of high intensity.* Average of experiments with 2 subjects (A and V) by *central vision.*
Abscissa: The area of the retinal image of the square in sq. mm (Log. scale).
Ordinate: Min. separabile in min. of arc.
——— Threshold I.
– – – – Threshold II.

23 and 24), so that the theory about the influence of the intensity discrimination by objects illuminated from before was confirmed.

A review of the dependence of the min. separabile on the size of the square can be obtained by summarizing the results of the investigation as follows:

A. Central Vision.

The experimental results obtained by *Berger & Buchthal* (1938 a) and *Berger* (1939) have been confirmed in all essentials.

The average diameter of the functional units in the centre is abt. 6 times the width of a cone. (As a measure of the diameter we have the min. separabile for luminous squares of threshold brightness on unilluminated ground, when the retinal images of the squares are smaller than one cone).

The smallest functional units consist of one cone. (The diameter may be determined by the min. separabile for unilluminated squares on luminous ground of threshold brightness when the squares exceed a certain size).

When the object is illuminated from before the min. separabile is not a measure of the size of the functional units, but a function of the intensity discrimination of the eye.

B. Indirect Vision.

Both the average diameter of the functional units and the size of the smallest functional units increase with the eccentricity up to abt. 3° in the horizontal meridian and 2° in the vertical meridian. Outside this boundary the receptor units are no longer functionally separated in groups (functional units), but the nerve impulses must be assumed to spread through transversal pathways in the retina. Here the min. separabile will consequently always be a function of the intensity discrimination.

Chapter V

Experiments on the Physiological Resolving Power 2

EXPERIMENTS ON THE PERCEPTION OF MOVEMENT

When a diffraction disc performs an oscillating movement on the retina it will not be possible to recognize the movement as long as it takes place within the same functional unit. Only when a neighbouring unit is also stimulated by the diffraction disc, so that it is illuminated above its intensity threshold, will the movement be appreciable (conf. p. 37).

As a diffraction disc may be estimated to have a diameter of abt. 60" and the excursions of the eye due to its constant oscillating movements to be abt. 30" from a central position, the image will travel over an area with a diameter of 120". In order to make the movement discernable the diffraction disc must cover a distance corresponding to the diameter of the functional units less 120". In this way it should be possible to determine the size of the functional units experimentally by means of a moving luminous point. As mentioned in chapter IV the diameter of the functional units in the centre can, however, also be measured by the min. separabile for luminous squares of threshold brightness on unilluminated ground. It should therefore be interesting to carry through a comparison, in order to verify the theory advanced with regard to the perception of the movement of a point and the determination of the size of the functional units.

No comparative experiments on the min. separabile determined in the manner mentioned and on the perception of the movement of a luminous point are found in the literature. New experiments would therefore be desirable.

By means of the arrangement mentioned on p. 96 a luminous point could be made to oscillate. Two thresholds were

Table XII.

Experiments on the perception of movement compared with the minimum separabile.

The movable object: a luminous point of threshold brightness on unilluminated ground. Each threshold determined by 20 readings. The min. separabile determined by means of two luminous squares (40" by 40") of threshold brightness on unilluminated ground. Both experiments were carried out with the same two subjects (A and V).

	Central Vision:		*5° Eccentricity (retino-nasally)*	
	The length of the movement when the point		The length of the movement when the point	
	is just seen to move	*seems to be stationary*	*is just seen to move*	*seems to be stationary*
	Threshold I	Threshold II	Threshold I	Threshold II
Subject A	62"	32"	172"	140"
Subject V	64"	25"	170"	109"
Average:	63"	29"	171"	125"
Min. separabile, average	183"	102"	480"	327"

determined by these experiments, one of which, threshold I, was the smallest observable movement of the luminous point synchronous with the actual movements of the point (so that it was avoided that the subject registered apparent movements). Threshold II was the largest movement of the luminous point which could not be appreciated (conf. p. 102). The experiments confirmed that the frequency of the movement had — as it might be expected (vide footnote on p. 37) — no influence on the thresholds, when it was between 1.1 and 5 complete movements per second. The experiments were performed with a frequency of 2 or 3 with two subjects (A and V). Each threshold was determined by 20 readings divided in 4 series.

In addition to the experiments by central vision corresponding experiments were carried out at an eccentricity of 5° retino-nasally in order to obtain also by indirect vision a comparison of the perception of movement with the min. separabile.

Table XII gives a juxtaposition of the results of the experiments and the min. separabile for the same subjects.

It appears from these results that by central vision the average value of the min. separabile (threshold I) is abt. 120" above threshold I from the experiment on perception of movement. The result confirms the theoretical calculation, so that it is possible, as maintained in chapter I, to regard the perception of movement as a measure of the physiological resolving power under the experimental conditions described. (Threshold II is not taken into consideration as it is not possible to correlate it to the retinal structure).

At an eccentricity of 5° the difference between the thresholds of the two functions is abt. 300", i. e. 180" larger than calculated. The fact that the physiological resolving power at an eccentricity of 5° is better when measured by the perception of movement than by the min. separabile must (as dealt with in details on p. 69) be explained by the dispersion of the nerve impulses (conf. that the dependence of the min. separabile on the size of the square must be explained in the same way at the same eccentricity (p. 139)).

Chapter VI

Experiments on the Physiological Resolving Power 3

EXPERIMENTS ON FORM SENSE

Through experiments by *Berger & Buchthal* (1938 b) it is confirmed that the form sense — as the min. separabile — is dependent on the structure of the retina. The relationship between the form sense and the retinal mosaic does not, however, seem to be so simple that the shape of the test object is of no importance. When f. inst. the form sense is measured by the minimum angular size of a certain regular polygon which allows perception of form, the threshold determined will be dependent on the number of sides of the polygon, especially if this is > 4.

Records of systematic observations on the form sense by indirect vision do not exist, so that it would be desirable to carry out such experiments. As a basis for the investigation it would be natural to verify *Berger & Buchthal's* results by central vision. By correlating the numerical experimental results to the average size of the functional units it should be possible to determine whether the form sense — as assumed (vide p. 41) — may be taken as a measure of the physiological resolving power.

In addition to the experiments on the dependence of the form sense on the retinal eccentricity the influence of the retinal illumination was examined, in order further to substantiate the theory about the alternating activity of the funcional units (vide p. 34).

The test object used was an equilateral triangle or a square, luminous on unilluminated ground (vide p. 98 as to details). Two thresholds were determined by these experiments. Threshold I represented the minimum length of side of the test

polygon at which it was possible to recognize its angularity. Threshold II represented the maximum length of side at which it was just impossible to recognize the angularity of the polygon (conf. p. 102).

A. Form Sense at Various Retinal Eccentricities.

Experiments were carried out with triangular and square test objects in the same sitting by central vision and with the square object at the following eccentricities: 10', 20', 30', 1°, 2°, 3° and 5° retino-nasally. The light intensity of the test object was adjusted at threshold brightness in these experiments. The result of the experiments was that the length of the sides of triangle and square must be 6.1' and 6.9' respectively, in order to make their angularity observable by central vision, i.e. 2.4 and 3.2 times the min. separabile for luminous points (threshold I) for the same subjects.

By indirect vision (vide fig. 25) both thresholds increase uniformly with the eccentricity, slightly less than the min. separabile, however. At an eccentricity of 3° threshold I is thus still 2.3 times the min. separabile, but at 5° the ratio is 1.8.

The fact, that it is possible to recognize the angularity of an equilateral triangle having a smaller length of side than the smallest recognizable square, indicates that the form sense is not exclusively determined by the length of side, when the object is a polygon, but that another factor, probably the size of the angles of the polygon, plays a rôle, as already mentioned by *Guillery* (1899). Centrally and up to an eccentricity of abt. 3° the absolute figures for the thresholds are larger than twice the min. separabile for luminous points, i.e. larger than twice the mean diameter of the functional units. That the same ratio at an eccentricity of 5° becomes less than 2 may be due to the fact that at this eccentricity there are no more funcional units, and the form sense therefore deteriorates.

The result of the experiment is thus that the form sense (as assumed on p. 41) is essentially a function of the fineness of the retinal

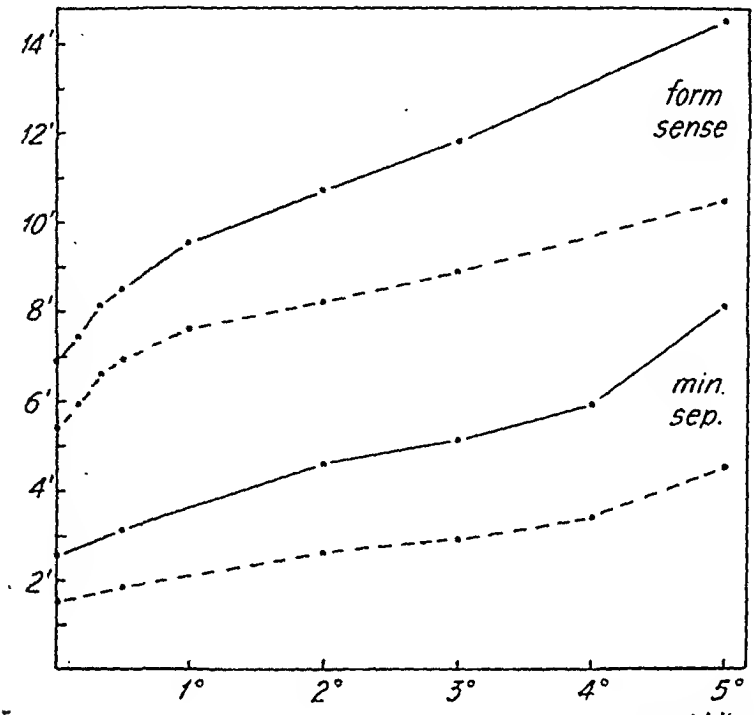


Fig. 25.

Form sense at various retinal eccentricities in juxtaposition with the minimum separabile for luminous points for the same two subjects (A and V).
Object: Luminous square of threshold brightness on unilluminated ground.
Abscissa: Eccentricity in degrees.
Ordinate: The length of the side of the square and the min. separabile both in min. of arc.
——— Threshold I.
– – – – Threshold II.

mosaic, when the function is measured by the minimum length of side of a polygon of few sides the angularity of which is just recognizable. On account of the incalculable influence of the number of sides the dimensions of the functional units can only be determined approximately through experiments on the form sense.

The accuracy of the experiments.

The accuracy of the threshold determinations was examined by means of special experiments with 20 readings of each threshold divided in 4 series, just as it was done in connection

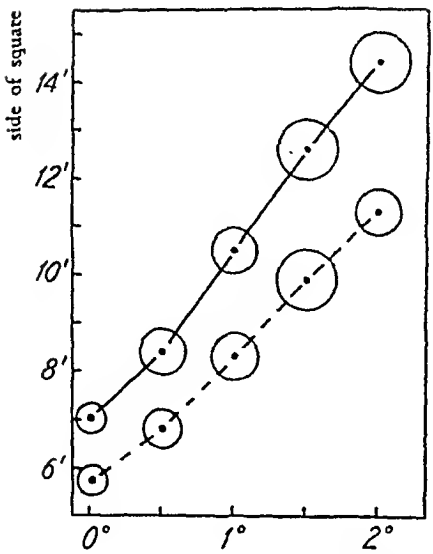


Fig. 26.

Form sense at various retinal eccentricities. *Investigation on the accuracy of the measurements.* Average for 3 subjects (G, M, S).
Object: Luminous square of threshold brightness on unilluminated ground. Round each point of the curve a circle has been drawn, the radius of which represents the dispersion of the threshold readings measured in min. of arc.
Abscissa: Eccentricity retino-nasally, in degrees.
Ordinate: Length of side of the square in min. of arc.
——— Threshold I.
– – – – Threshold II.

with the experiments on the min. separabile (p. 110). Table XIII records the dispersion of the readings and the variation coefficient calculated in the same way as in chapter IV (p. 111). On fig. 26 the average results for 3 subjects are shown. It appears that the dispersion increases with increasing retinal eccentricity, but that the percentage accuracy over the entire area examined from 0 to an eccentricity of 2° is constantly abt. 5 for both thresholds. This accuracy, which must be considered satisfactory, is of the same magnitude as the accuracy with which the min. separabile is determined.

Table XIII.

Dispersion and variation coefficient in experiments on form sense from 0 to an eccentricity of 2° retino-nasally.

Test object: Luminous square of threshold brightness on unilluminated ground.

Length of the side of the square in min. of arc. (Average for two days).

Threshold I	Subject	0	30'	1°	1°30'	2°	Number of readings
Threshold I	G.	8.4'	9.3'	11.5'	14.6'	17.2'	20
	M.	5.5'	6.9'	8.3'	9.5'	10.4'	20
	S.	7.2'	9.0'	11.7'	13.6'	15.7'	20
Average		7.0'	8.4'	10.5'	12.6'	14.4'	60
Threshold II	G.	6.5'	7.5'	8.8'	11.2'	13.4'	20
	M.	4.9'	6.0'	7.5'	8.4'	9.0'	20
	S.	5.8'	6.9'	8.7'	10.0'	11.4'	20
Average		5.7'	6.8'	8.3'	9.9'	11.3'	60
Dispersion (S) of the threshold determinations.							
Threshold I	G.	0.2'	0.5'	0.5'	0.5'	0.5'	20
	M.	0.3'	0.4'	0.4'	0.6'	0.5'	20
	S.	0.5'	0.7'	0.7'	0.7'	0.8'	20
Average		0.3'	0.5'	0.5'	0.6'	0.6'	60
Threshold II	G.	0.3'	0.4'	0.7'	0.6'	0.7'	20
	M.	0.2'	0.4'	0.3'	0.6'	0.4'	20
	S.	0.3'	0.4'	0.6'	0.6'	0.4'	20
Average		0.3'	0.4'	0.5'	0.6'	0.5'	60
Coefficient of variation (K).							
Threshold I	G.	3	5	4	4	3	20
	M.	6	6	4	6	4	20
	S.	7	8	6	5	5	20
Average		5	6	5	5	4	60
Threshold II	G.	5	5	8	5	4	20
	M.	5	6	4	7	4	20
	S.	6	6	6	6	3	20
Average		5	6	6	6	3	60

B. The Dependence of the Form Sense on the Retinal Illumination.

A detailed account has previously (pp. 35 and 41) been given of the experiments by *Berger & Buchthal* (1938 b) on form sense at varying retinal illumination and of the explanation of the results based on the theory about the alternating activity of the functional units. Here it shall only be mentioned that the experiments show that the form sense is better at high retinal illumination (abt. 2 lux) than by threshold brightness (abt. 0.025 lux). The dependence is the same, irrespective of whether the object is a luminous polygon on unilluminated ground or an unilluminated polygon on luminous ground.

As the variation of the form sense with the illumination has not previously been investigated by indirect vision, such experiments were carried out at an eccentricity of 5° retinonasally with a luminous square on unilluminated ground as test object. It was also found desirable to verify *Berger & Buchthal's* results in the centre over a larger intensity range (from threshold brightness to 100 lux on the retina). It appears from fig. 27 that by central vision both the thresholds examined decreased uniformly with increasing illumination. At an eccentricity of 5° the same happens, with the modification, however, that at intense illumination the curve rises again sharply.

That the form sense by central vision improves with increasing retinal illumination may be due to the fact that an increased dispersion in the refractive media increases the perceived size of a luminous object (conf. p. 31). According to *Berger & Buchthal* the form sense varies, however, in the same way with the illumination, when it is a case of unilluminated figures on luminous ground. The improvement of the form sense with increasing illumination of relatively low intensity can therefore only be explained by the assumption that the number of active functional units per unit of area increases. When the light intensity of the object increases so much that the hitherto un-

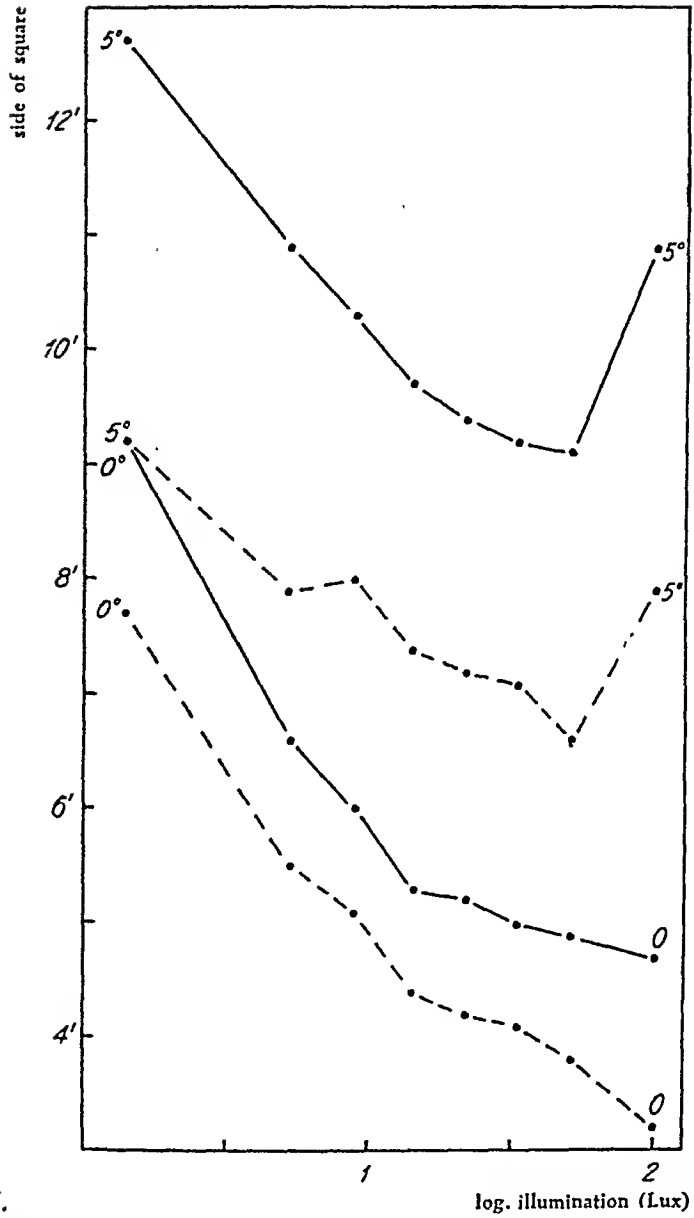


Fig. 27.

Form sense by varying retinal illumination, centrally and at an eccentricity of 5° retino-nasally. Average for 2 subjects (A and V).
Object: Luminous square on unilluminated ground.
Abscissa: The logarithm of the retinal illumination (in lux).
Ordinate: The length of the side of the square in min. of arc.
——— Threshold I.
– – – – Threshold II.

stimulated parts of the retina are stimulated above their intensity threshold, it should be possible for the intensity discrimination to

make itself felt. (conf. p. 11). It is not possible, however, to estimate the influence of this possibility on the form sense.

The experiments carried out at an eccentricity of 5° should, with regard to the declining part of the curve, probably be explained in the same way as by central vision. It is difficult to explain why the form sense again deteriorates by very intense retinal illumination. Perhaps the phenomenon is due to the fact that the strong dispersion of the light obliterates the details of the figures (the verteces).

Chapter VII

Experiments on Complex Functions 1

EXPERIMENTS ON THE ALIGNING POWER

If two halves of a rectilinear contour are displaced on the retina in relation to each other as the hair lines of a vernier, then the smallest recognizable displacement, the displacement threshold, will be a measure of the aligning power. On page 43 it was pointed out, that the condition, which must be fulfilled before the displacement becomes visible, is that the illumination of one half of a long straight row of functional units differ so much from that of the other half (the row is imagined to be divided transversely) that a difference in the retinal illumination is recognizable. The aligning power thus becomes a function of the simultaneous intensity discrimination. As the displacement threshold by the same discrimination factor must be assumed to be smaller by small functional units than by large ones, it is moreover a function of the physiological resolving power (the fineness of the retinal mosaic) and thus a complex function.

Especially on account of the complex physiological basis of the aligning power it was thought to be of interest to investigate its dependence on the retinal eccentricity. An object consisting of a narrow luminous slit of threshold brightness in unilluminated ground (vide p. 99) was used for the experiments. The thresholds determined by the experiments were in the first place the displacement threshold (threshold I) and secondly the maximum displacement at which the half parts of the slit were seen to be in alignment (threshold II).

In addition to the experiments performed at various eccentricities others were carried out through which the displacement threshold was determined for slits of decreasing length in order to examine the transition of the complex function to a simple one (the min. separabile for luminous points).

A. The Aligning Power at Various Retinal Eccentricities.

The test object for the experiments on the dependence of the aligning power on the retinal eccentricity consisted in a luminous slit 50" by 2·18'20" of slightly more than threshold brightness, placed at right angles to the meridian examined.

In the centre the displacement threshold for three subjects was determined to be 23", 25" and 34" respectively. The fact

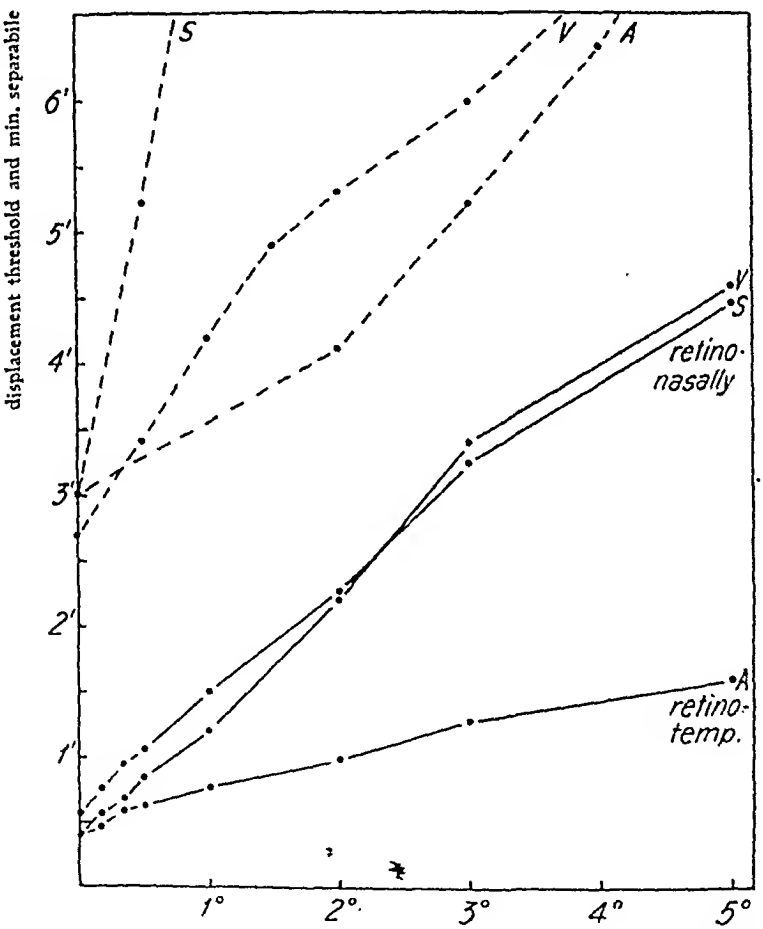


Fig. 28.

The displacement threshold at various retinal eccentricities in juxtaposition with the minimum separabile for luminous points for the same three subjects (A, V, S).
Object: Luminous slit (50" by 2·18'20") of threshold brightness on unilluminated ground.
Abscissa: Eccentricity in degrees.
Ordinate: Displacement threshold and min. separabile in min. of arc.
——— Displacement threshold.
– – – – Min. separabile.

that these figures are somewhat higher than those found by other authors (vide table IV, p. 65) can probably be explained by the relatively small length of the slit used in these experiments.

In addition to being determined in the centre the displacement threshold was also determined at eccentricities of 10', 20', 30', 1°, 2° and 5°. It can be seen from fig. 28 that in the whole of the area examined the displacement threshold increases uniformly with increasing eccentricity, but less rapidly than the min. separabile. The rise of the curve is smooth without any break corresponding to the border of the rod-free area or the fovea, just as it is the case for the min. separabile. The fact that the displacement threshold does not increase with the eccentricity to the same extent as the min. separabile is in conformity with the assumption previously mentioned, that the aligning power is not exclusively a function of the physiological resolving power. As it will appear from the following chapter (p. 169) the dependence found seems to suggest that the aligning power is a complex function of both the physiological resolving power and the intensity discrimination.

The accuracy of the experiments.

In order to be able to judge the reliability of the numerical experimental results, experiments were carried out on the accuracy of the threshold determinations centrally and at an eccentricity of 5°. The test object was the same as the one used in the above mentioned experiments. For each threshold 20 readings divided in 4 series were taken. The mean (M) of the 20 readings, the calculated standard deviation δ and the variation coefficient $k = \frac{\delta \cdot 100}{M}$ are recorded in table XIV.

It appears from the table that while the percentage accuracy of threshold I both centrally and at an eccentricity of 5° is abt. 11, it is not nearly so good for threshold II (15 to 54). For this reason threshold II has not been included in the discussion of the results of the remaining experiments. Thre-

Table XIV.

The standard deviation and variation coefficient by experiments on the aligning power, centrally and at an eccentricity of 5°. Object: Luminous slit, 50" by 2·18'20" of threshold brightness on unilluminated ground.

The displacement of one half of the slit measured in seconds of arc.

	Subject	0	5°	Meridian	Number of readings
Threshold I:	A.	19"	114"	retino-temporal	20
	V.	20"	268"	retino-nasal	20
	S.	37"	332"	retino-nasal	20
Threshold II:	A.	4"	49"	retino-temporal	20
	V.	*)	28"	retino-nasal	20
	S.	18"	140"	retino-nasal	20

*) unreadable

Standard deviation (σ).

Threshold I:	A.	2.2"	15"	retino-temporal	20
	V.	2.4"	29"	retino-nasal	20
	S.	3.0"	36"	retino-nasal	20
Threshold II:	A.	2.1"	13"	retino-temporal	20
	V.		28"	retino-nasal	20
	S.	2.7"	30"	retino-nasal	20

Variation coefficient (k).

Threshold I:	A.	11	14	retino-temporal	20
	V.	12	11	retino-nasal	20
	S.	8	11	retino-nasal	20
Threshold II:	A.	54	26	retino-temporal	20
	V.		15	retino-nasal	20
	S.	15	21	retino-nasal	20

shold II has, however, always been determined in order to obtain a convenient starting point for the determination of threshold I. It thus seems that the determination of the just recognizable displacement of the

two halves of the slit is easier (more certain) than the determination of the point where they are still seen to be in alignment.

B. The Dependence of the Displacement Threshold on the Length of the Slit.

The only possible explanation of the fact, that it is possible to recognize a displacement of the retinal image which is smaller than the smallest functional units, is that the displacement threshold is in any case a function of the simultaneous intensity discrimination (conf. p. 43). The two narrow retinal areas the illumination of which is being compared are in alignment. As the intensity discrimination increases with increasing size of object (vide p. 9) it might be expected that the displacement threshold varied correspondingly. However, the two halves of the slit must have a certain length in order to be seen as lines by the subject. If they become so short that two luminous points are all that remains, then the minimum appreciable displacement will be equal to the min. separabile. As this function is a measure of the physiological resolving power, when determined for luminous points of threshold brightness on unilluminated ground, a curve representing the dependence of the displacement threshold on the length of the slit will illustrate the transition from a simple function (the min. separabile) to a complex function (the displacement threshold).

The test object was a luminous slit, 20" wide, the length of which varied from 2·20" to 2·20'. Before dealing with the experimental results a description shall be given of the transition between the min. separabile and the displacement threshold, as seen by the subject by central vision. In the case of the two shortest slits (2·20" and 2·60") the minimum discernable displacement is equal to the min. separabile. When the slit increases in length (2·100") it is seen as a vertical line when the two halves are in alignment, but when a displacement manifests itself the line will appear inclined. The two halves of the slit will only appear as two independent lines,

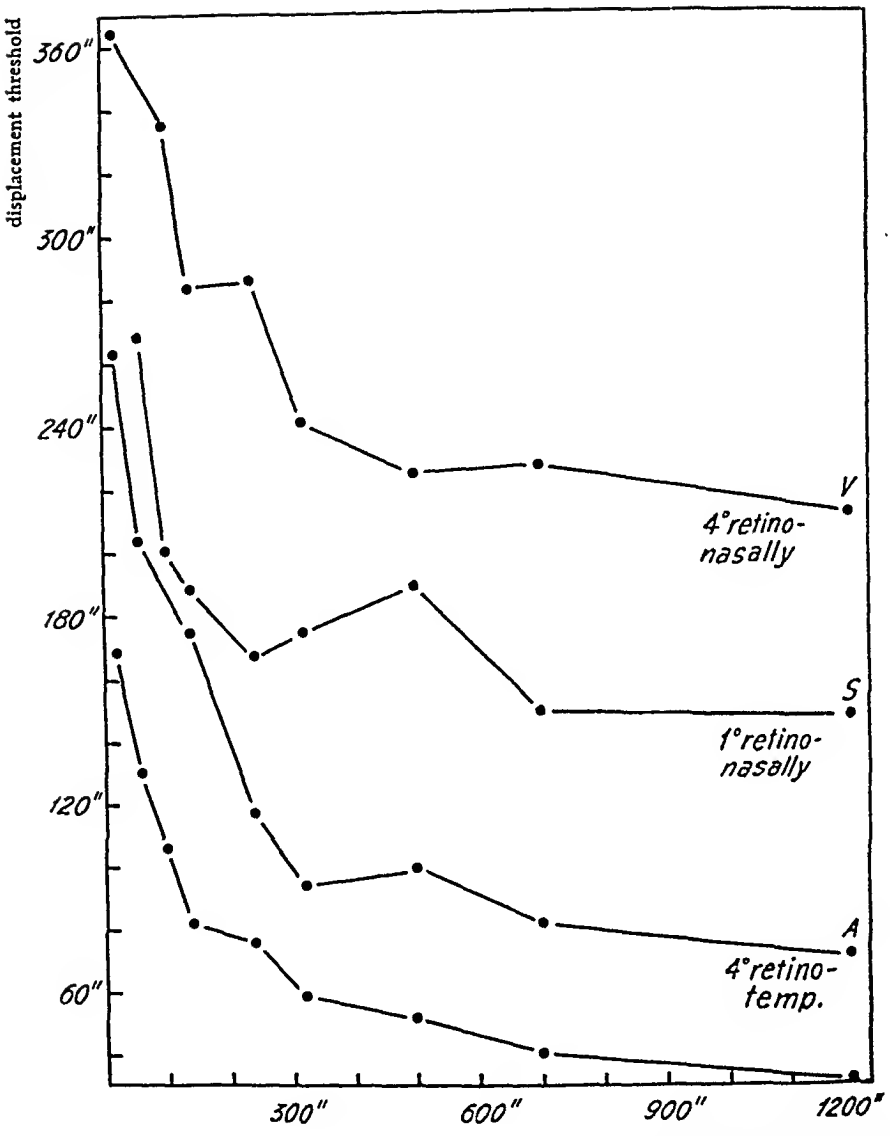


Fig. 29.

The dependence of the displacement threshold on the length of the slit, centrally and at eccentricities of 4° or 1°. The lowest curve is an average curve for 3 subjects (A, V, S), representing the dependence by central vision.
Object: Luminous slit, 20" wide, on unilluminated ground.
Abscissa: Length of each half part of the slit in sec. of arc.
Ordinate: Displacement threshold in sec. of arc.

which are displaced in relation to each other, when they have reached a greather length (2·320").

The experimental results appear from fig. 29. It appears that the displacement threshold decreases,

when the length of the slit increases, first rapidly until each slit is seen as a line, and then at a steadily decreasing rate. By similar experiments it was shown that the displacement threshold varies in the same way at eccentricities of 1° and 4° as it does centrally. On account of the complexity of the aligning power it will be impossible to explain the course of the curve in details. (vide p. 174, however).

Chapter VIII

Experiments on Complex Functions 2

EXPERIMENTS ON DISCRIMINATION OF THICKNESS OF LINE

When the thickness of one half part of a line or slit is different from the other, then the smallest perceptible difference in thickness will be a measure of the function which in this book has been termed the discrimination of thickness of line. According to the theoretical division of the visual sense into partial functions the discrimination of thickness of line should be a simple function of the simultaneous intensity discrimination when the object is above a certain size (vide p. 44). This assumption is also confirmed by the fact that if the light intensity of one half of a luminous slit is reduced, that particular half part will appear narrower than the other. In spite of the similar appearance of the two objects the discrimination of thickness of line rests on a physiological basis quite different from that of the aligning power (vide p. 43).

The experimental apparatus, which is described in details on p. 99, allowed for experiments with a luminous slit in an unilluminated ground as well as an unilluminated slit in a white ground illuminated from before. Two thresholds were determined through these experiments, threshold I being the smallest discriminable increase in thickness of one half of the slit, and threshold II the smallest observable decrease in thickness (conf. p. 102).

As mentioned on page 66 *Adler & Meyer* (1935) found that the discrimination of thickness of line is equally acute everywhere in the fovea, but deteriorates rapidly from the border of the fovea and outwards. This suggests the existence of a »physiological fovea«, the size of which corresponds to the anatomical (up to an eccentricity of 42'), but this is directly

contradictory to the dependence of the other functions on the retinal eccentricity which has been substantiated in the preceding chapters. It was therefore endeavoured to repeat *Adler & Meyer's* experiments. The influence of the retinal illumination and the dimensions of the objects on the thresholds read was also investigated.

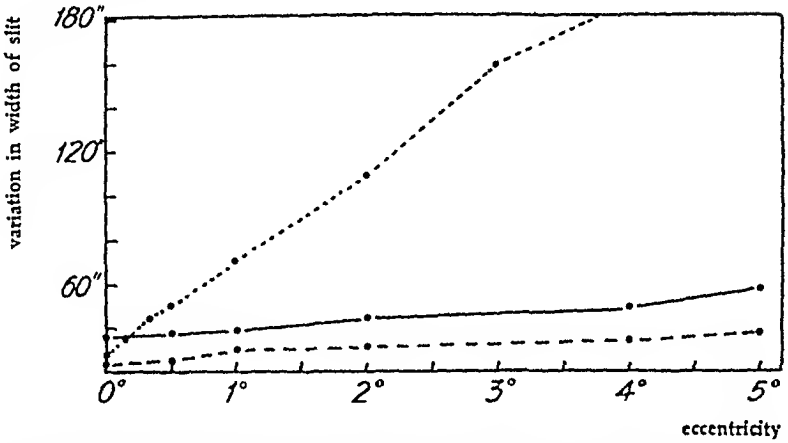
A. Discrimination of Thickness of Line at Various Retinal Eccentricities.

The aim of the experiments was — as mentioned above — to verify *Adler & Meyer's* theory about a »physiological fovea« with an outer boundary at an eccentricity of 42'. The object used by the two authors for their experiments was a dark slit in a white screen illuminated from before by flashes of light of a duration of 0.16 seconds. The length of the slit was 2·11'40", the width of the stationary half of the slit 35". As the first orientating experiments did not give results confirming those of *Adler & Meyer*, it was found desirable to investigate the dependence on the retinal eccentricity under various conditions, in the first place with a luminous slit in unilluminated ground in the second place with an unilluminated slit in a constantly illuminated white ground, and in the third place with the same object momentarily illuminated (vide p. 89). The unilluminated slit was of the same dimensions as the object used by *Adler & Meyer*.

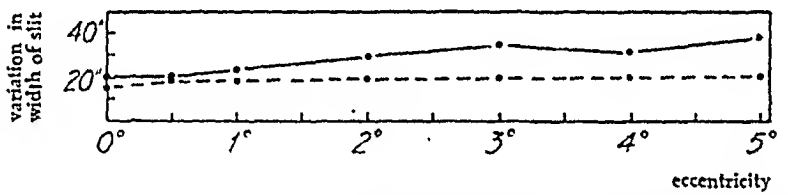
At the experiments with a luminous slit in an unilluminated ground the length of the slit was 2·15', the width of the stationary half of the slit 1'. In the area from the centre to an eccentricity of 5° the increase of both thresholds (vide fig. 30 a) with increasing eccentricity is only slight, there is no indication of a »physiological fovea«. The experiments with an unilluminated slit in white ground yield the same result both by continuous illumination and by an exposure time of 0.07 seconds (vide fig. 30 b and c and table XV in which *Adler & Meyer's* results are recorded).

Fig. 30.

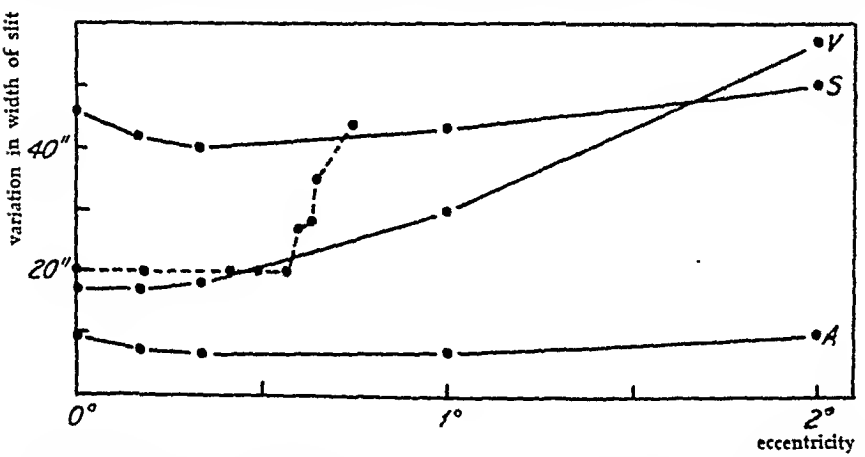
Discrimination of thickness of line at various retinal eccentricities.
Abscissa: Eccentricity in degrees.
Ordinate: Variation in the width of the slit in sec. of arc.
——— Threshold I.
– – – – Threshold II.



a. *Luminous slit in unilluminated ground.* Dimensions of the slit: length: 2·15′, width: 60″. Continuous illumination. Average for 3 subjects (A, G, S).
- - - - - - Displacement threshold.



b. *Unilluminated slit in white ground. Continuous illumination from before.* Dimensions of the slit: length: 2·11′ 40″, width: 35″. Average for 3 subjects (A, V, S).



c. *Unilluminated slit in white ground, momentary illumination* (0.37 sec.). Dimensions of the slit as in *b*. Curves for 3 subjects (A, V and S). In order to make the graph more perspicuous threshold II has not been included.
- - - - - - Threshold I according to experiments by *Adler & Meyer* (1935).

Table XV.

Discrimination of thickness of line at various eccentricities. According to experiments by Adler & Meyer (1935). Object: dark slit (35" wide, 2·11'40" long) on white ground, illuminated momentarily (0.16 sec.).

Eccentricity retino-nasally:	0	11'	25'	29'	34'	36'	38'	39'	45'
Threshold I:	20"	20"	20"	20"	20"	27"	28"	35"	44"

It cannot be explained with certainty why the dependence on the retinal eccentricity of the discrimination of thickness of line is much less pronounced than that of the aligning power (vide fig. 30 a). It shall only be suggested that, as the min. separabile for luminous points is a function of the physiological resolving power, while the discrimination of thickness of line, under the conditions mentioned, is a function of the intensity discrimination, the dependence on the eccentricity of the aligning power, which, according to chapter VII, is a complex function of both, will probably lie between that of the two other functions.

As the discrimination of thickness of line is a measure of the differential threshold, it must be possible to determine the size of the latter in per cent as the ratio between the variation of the thickness of the line and the width of the constant half of the slit. According to this the threshold for discriminable increase of intensity is calculated from the results of the experiments with a luminous slit in unilluminated ground to have an average value of 60 per cent centrally and 95 per cent at an eccentricity of 5°. From a corresponding experiment (p. 171) the figures are found to be 52 per cent and 121 per cent, that is roughly a doubling of the differential threshold. In this connection it may be stated that through experiments with *Masson*'s discs *Dobrowolsky & Gaine* (1876) and *Bjerrum* (1882[105]) (vide p. 60) found the differential threshold at an eccentricity of 5° to be twice as large as the differential threshold centrally.

Before discussing the discrepancy between *Adler & Meyer's*

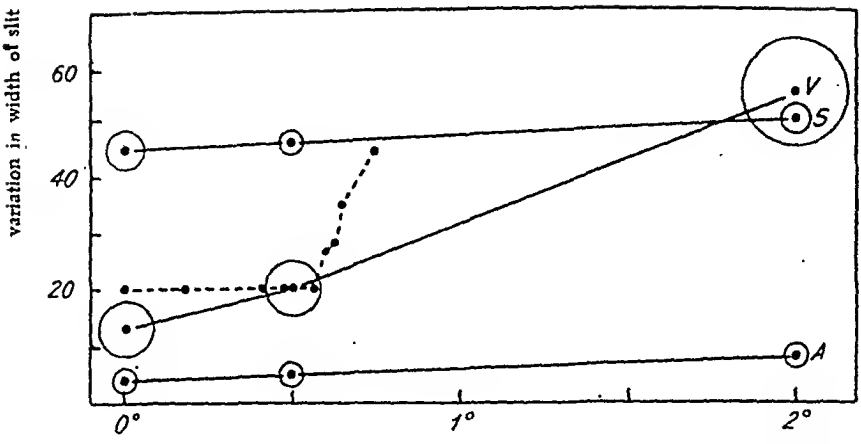


Fig. 31.

Discrimination of thickness of line at various retinal eccentricities. *Experiments on the accuracy of the experiments.*
Object: Unilluminated slit in white ground, momentarily illuminated (0.07 seconds).
Dimensions of the slit: Length: 2·11′ 40″, width: 35″. Curves for three subjects (A, V and S).
To make the graph more perspicuous threshold II has not been included.
Round each point of the curve a circle has been drawn, the radius of which denotes the dispersion of the threshold readings measured in seconds of arc.
Abscissa: Eccentricity in degrees.
Ordinate: Variation in width of slit in seconds of arc.
——— Threshold I.
– – – – Threshold I according to experiments by *Adler & Meyer* (1935).

results and those of the present experiments, a description will be given of experiments on the accuracy of the determination of thickness of line.

The accuracy of the experiments.

With the same objects as those used in the experiments described above, other experiments on the accuracy of the threshold determinations were carried out. 20 readings of each threshold divided in four series were performed, centrally as well as at an eccentricity of 5°, with luminous slits. Corresponding experiments were performed with momentary illumination (0.07 sec.) of a white ground with an unilluminated slit, centrally and at eccentricities of 30′ and 2°. The number of readings was 20 as in the above mentioned experiment, but the readings were divided in four series carried out in the course of two days.

The experimental results appear from table XVI and fig. 31.

In proportion to the small amount by which the two

thresholds increase with the eccentricity the dispersion of the results is rather large and the percentage accuracy on the whole not very good. All the results from experiments on the discrimination of thickness of

Table XVI.

Dispersion and variation coefficient by experiments on the discrimination of thickness of line, centrally and by indirect vision.

a. *Luminous slit in unilluminated ground* (length of the slit: 2·15', width: 60").

Increase (threshold I) and decrease (threshold II) of the width of one half of the slit, measured in seconds of arc.

	Subject	0°	5	Meridian	Number of readings
Threshold I	A.	24"	44"	retino-temporal	20
	V.	37"	91"	retino-nasal	20
	S.	32"	83"	retino-nasal	20
Threshold II	A.	11"	20"	retino-temporal	20
	V.	16"	35"	retino-nasal	20
	S.	26"	34"	retino-nasal	20
Standard deviation (σ).					
Threshold I	A.	4.8"	11"	retino-temporal	20
	V.	7.3"	13"	retino-nasal	20
	S.	9.1"	22"	retino-nasal	20
Threshold II	A.	3.5"	3.6"	retino-temporal	20
	V.	9.7"	4.3"	retino-nasal	20
	S.	3.4"	4.0"	retino-nasal	20
Variation coefficient (k).					
Threshold I	A.	20	25	retino-temporal	20
	V.	20	14	retino-nasal	20
	S.	28	27	retino-nasal	20
Threshold II	A.	32	18	retino-temporal	20
	V.	61	12	retino-nasal	20
	S.	13	12	retino-nasal	20

b. *Unilluminated slit in white ground illuminated from before. Momentary illumination* (0.07 sec.). The length of the slit 2·11'40", width: 35".

The increase (threshold I) and decrease (threshold II) of the width of one half part of the slit, measured in seconds of arc.

	Subject	0°	30'	2°	Meridian	Number of readings
Threshold I	A.	4"	5"	8"	retino-temporal	20
	V.	13"	20"	55"	retino-nasal	20
	S.	45"	46"	50"	retino-nasal	20
Threshold II	A.	14"	14"	12"	retino-temporal	20
	V.	20"	22"		retino-nasal	20
	S.	24"	26"	29"	retino-nasal	20
Dispersion (S) of the threshold determinations.						
Threshold I	A.	2.1"	2.1"	2.2"	retino-temporal	20
	V.	5.0"	4.7"	9.2"	retino-nasal	20
	S.	3.3"	1.8"	2.7"	retino-nasal	20
Threshold II	A.	1.7"	2.2"	2.0"	retino-temporal	20
	V.	2.9"	3.3"		retino-nasal	20
	S.	1.8"	1.3"	1.5"	retino-nasal	20
Variation coefficient (K).						
Threshold I	A.	56	44	29	retino-temporal	20
	V.	51	25	16	retino-nasal	20
	S.	7	4	5	retino-nasal	20
Threshold II	A.	12	22	16	retino-temporal	20
	V.	14	16		retino-nasal	20
	S.	8	5	5	retino-nasal	20

line must therefore be taken with some reservation.

An explanation of the difference between the results of *Adler & Meyer* and those of the present experiments cannot be given with certainty. The difference in exposure time cannot be of any importance. It would be natural to ascribe the difference to the poor accuracy of the

threshold determinations. If the subject's eye has not been properly light-adapted, this may also have a certain influence, and it does not appear from *Adler & Meyer's* paper, whether this was the case or not (ref. p. 66).

B. The Influence of the Retinal Illumination on the Discrimination of Thickness of Line.

If it is possible to regard the discrimination of thickness of line as a measure of the simultaneous intensity discrimination, the function must be relatively independent of the light intensity.

By means of an object consisting in a luminous slit (width: 60", length: 2·11'40") in unilluminated ground it was therefore endeavoured to investigate the influence of the brightness of the object on the discrimination of thickness of line within a range of intensities from threshold brightness (1.4 lux on the retina) to pronounced glare (100 lux). It appears from fig. 32 that centrally the determination of thickness of line is independent of the illumination except by glare, where the thresholds examined increase slightly. By indirect vision (5° ecc.) the thresholds first decrease slightly, then remain constant and again increase when glare occurs.

The course of the curve found corresponds to the one which characterizes the differential threshold (mentioned on page 7) and thus confirms that under the given conditions the discrimination of thickness of line is a measure of the intensity discrimination.

C. The Influence of the Dimensions of the Object on the Discrimination of Thickness of Line.

1. The Influence of the Length of the Line.

In experiments on the discrimination of thickness of line it is an absolutely necessary condition that each half part of the test object is of such a length that it can be recognized as

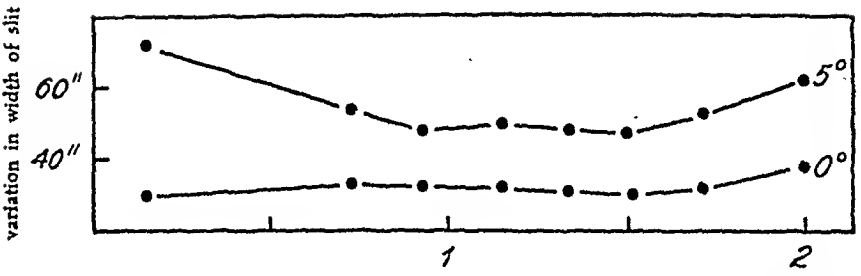


Fig. 32.

Discrimination of thickness of line at varying retinal illumination, centrally and at an eccentricity of 5° (retino-nasally).
Object: Luminous slit (2·15′ long, 60″ wide) in unilluminated ground. Average for 2 subjects (A and V). To make the graph more perspicuous threshold II has not been included.
Abscissa: The logarithm of the retinal illumination (in lux).
Ordinate: Variation of the width of the slit in sec. of arc.

a line or slit by the subject. If the slit is shorter, an increase in »thickness« will manifest itself in the appearance of a horizontal line or an obtuse angular figure, i. e. the function measured will be a measure of the form sense rather than the discrimination of thickness of line. If the discrimination of thickness of line was closely related to the aligning power, it was to be expected that the former improved with increasing length of slit (conf. p. 164). If, however, the discrimination of thickness of line is only a measure of the intensity discrimination the influence of the length of the slit is unpredictable, as the few experiments which have been carried out on the variation of the simultaneous differential threshold with the size of the object have been performed with a circular object, the brightness of which was compared with that of its surrounds (vide p. 9).

The average results of experiments with three subjects, by central vision and with an object consisting of a 60" wide luminous slit appear from fig. 33. It will be seen that the length of the slit has no influence when the length is more than abt. 4 times the width. The experiment thus confirms that the discrimination of thickness of line, in contradistinction to the aligning power, is to a great extent independent of the length of the line. This result indicates that the differential threshold, when determined for

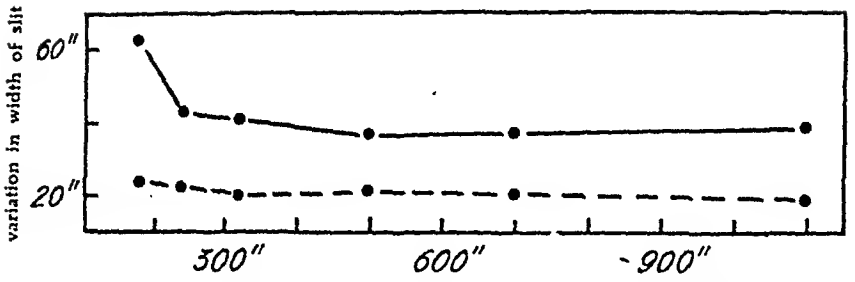


Fig. 33.

The influence of the length of the line on the discrimination of thickness of line by central vision.
Object: Luminous slit in unilluminated ground. The width of the stationary half part of the slit: 60". Average for 3 subjects (G, V, S).
Abscissa: Length of each half part of the slit in sec. of arc.
Ordinate: Variation of width of slit in sec. of arc.
——— Threshold I.
– – – Threshold II.

two narrow rectangles in alignment with eachother, will react in the same way.

2. The Importance of the Width of the Line.

If the thresholds determined in connection with discrimination of thickness of line consisted in an appreciation of the displacement of a contour, as by the determination of the aligning power, the width of the line (slit) would probably be of slight importance. If, however, the threshold is a function of the intensity discrimination, a certain interdependence between the length of the boundary line separating the two »photometer fields« (the width of the slit) and the thresholds examined would probably be found.

By experiments with a luminous slit, 2·18'20" long, in unilluminated ground both thresholds were found to be dependent on the width of the stationary half of the slit by central vision (fig. 34). The thresholds increase with increasing width of the slit, not linearly, but gradually less and less. The smallest discriminable increase or decrease of the width of one half of the slit is thus a gradually decreasing fraction of the width of the stationary slit. Expressed in per cent threshold I decreases from about 62 to 26 per cent. If these figures are taken to be a measure of the intensity discrimination, it may be concluded from the experiments that the intensity discrimination within the area examined increases

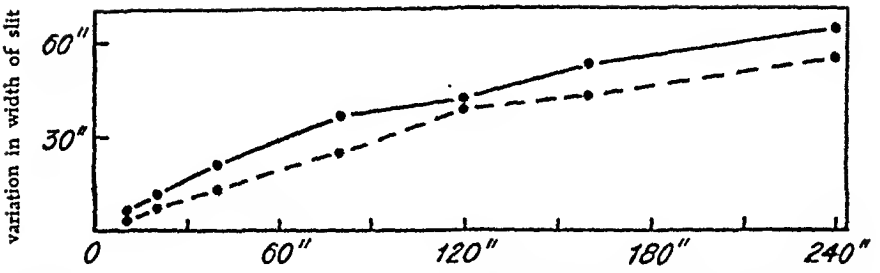


Fig. 34.

The influence of the width of the slit on the discrimination of thickness of line by central vision.
Object: Luminous slit (2·18′ 20″ long) in unilluminated ground. Average for 3 subjects (G, V, S).
Abscissa: Length of each half part of the slit in sec. of arc.
Ordinate: Variation of width of slit in sec. of arc.
——— Threshold I.
– – – – Threshold II.

with the stimulated retinal area. (conf. p. 9). It is not, however, certain that the discrimination of thickness of line by maximum thickness is a function of the intensity discrimination exclusively, as threshold I (the smallest recognisable increase in thickness) is here so large (60") that it may be possible to estimate the absolute width of the slit.

The influence of the dimensions of the line on the discrimination of thickness of line thus confirms — as do the experiments on the influence of the retinal eccentricity and illumination — the theory that under suitable experimental conditions the discrimination of thickness of line is a function of the simultaneous intensity discrimination.

Chapter IX

Appendix

EXPERIMENTS ON TROXLER'S PHENOMENON

It is generally assumed that the sensitivity of the retinal functional units is constant. It has already been mentioned on pp. 34, 131 and 156 that this does not seem to be the case, as various experiments cannot be explained except by resorting to a theory about an alternating activity of the functional units. In order further to expound this problem some experiments on *Troxler*'s phenomenon are recorded in the following.

A. Troxler's Phenomenon and the Theories Advanced in Explanation of it.

In the year 1804 the Swiss, *Ignaz Troxler,* recorded the observation that even if a steady fixation is maintained by monocular vision it will be possible with the indirect vision to observe the disappearance of small stationary objects, although their images do not fall on the blind spot of *Mariotte.* This experiment has been confirmed several times since then, e. g. by *Purkinje* (1823 [76, 78]) who observed that the fixation point itself could also disappear, and that the vanished object could reappear, even if the steady fixation was maintained. *Aubert* (1865 [103]) explained the phenomenon as a result of fatigue, while *Helmholtz* (1867 [364]) did not find it possible to give any satisfactory explanation. *Holth* (1896 [110]), who made an intensive study of *Troxler*'s phenomenon and other phenomena due to »gazing blindness«, found that the disappearance of larger objects by indirect vision could be avoided by intermittent blinking, probably because the eye performs small movements, so that the image falls on new, unfatigued elements. The

disappearance of points and quite small objects even under constant blinking could, according to *Holth,* only be explained by means of variations in the attention. With the experimental technique applied by *Holth* he had hardly any opportunity to observe the disappearance of objects seen centrally.

Without apparently having any knowledge of previous literature on this subject *V. Hensen* (1897) described *Troxler*'s phenomenon, when the object is a number of black or faintly luminous points seen centrally. If a certain point is fixated, some of the points — and sometimes the fixation point as well — will presently be seen to disappear, but soon after that they will reappear, while other points become invisible for a time. This alternation between visibility and invisibility of the points takes place quietly, but it is not possible for the subject to recognize any system. Under optimal experimental conditions especially by relatively low illumination, abt. ⅓ of the points disappear simultaneously.

As the alternation of the points takes place quietly and not at all by jerks, *Hensen* concludes that the phenomeon cannot be due to movements of the eye, but he assumes that the points disappear, when their retinal images fall on the spaces between the outer segments of the cones and thus on parts of the retina which is supposed to be insensitive. This explanation, which is also given by *Duke-Elder* (1938 [903]), can hardly be maintained, when the involuntary oscillating movements of the eye and the magnitude of the minimal retinal image are taken into consideration.

»The changing play of the details in the intrinsic light of the retina« has also been used as explanation of the phenomenon (*F. Klein*, 1911) without the actual solution of the problem having been brought any nearer.

Hofmann (1925 [67]) draws the attention to an interesting observation made during experiments with *Hensen's* point objects. When the subject gazes at the points for a considerable time, they will appear to change their shape, become indented and angular. Often two points side by side are seen in stead of one point.

It is further possible that the functional units gradually

become insensitive to the constant light stimulation by a kind of local adaptation (conf. p. 129). With regard to central vision, however, this theory does not conform with the fact that the points reappear on continued fixation (*Hofmann* 1925 [67]). The importance of local adaptation for indirect vision will be dealt with later on.

As *Troxler*'s phenomenon does not only appear by indirect vision, but also by central and paracentral vision it cannot — as proposed by *Holth* (1896 [124]) — be explained by variations in the attention which is especially attached to central vision (vide pp. 54 and 119) and can hardly be imagined to change according to a speckled pattern.

There is, however, another possible explanation which has not been pointed out before, namely the theory mentioned on pp. 34 and 156, according to which the retinal functional units display an alternating activity consisting of active and relatively refractory periods, it being assumed that the images of vanished points fall on refractory units. If the theory holds, *Troxler*'s phenomenon must be clearly dependent on the retinal illumination. It has been endeavoured to elucidate this problem in the following subsection.

When considering the problem more closely it will be seen that the theory about alternating activity can only explain the disappearance of luminous points directly, while it is necessary further to expound the theory with regard to dark points on luminous ground. It has previously (p. 107) been mentioned that a great number of the functional units in the centre of the fovea consist of numerous cones. If it is assumed that in addition to the alternating activity of the functional units the individual cones, of which they are made up, also have relatively refractory periods, the images of dark points may be supposed now and then to fall on such refractory cones. In that case other cones belonging to the same unit are perhaps in their active period, and the unit as such is stimulated. The retina will not register the dark point, it will be invisible as long as it falls on refractory cones.

It might accordingly be expected that on a luminous surface of threshold brightness dark spots corresponding to

inactive units would be seen. Even if actual, well-defined spots are not seen, such a surface appears somewhat inhomogenous, just as the luminous polygons of threshold brightness, used in the experiments on form sense and described in chapter VI, could often be seen to »loose« a vertex temporarily.

An objection might be raised against the theory about the alternating activity of the functional units, namely that it cannot explain the disappearance of large objects. To this it may be averred that relatively large objects only vanish by indirect vision, and that the corresponding functional units are much larger than in the centre of the retina, and that outside a certain limit there are no functional units at all, so that the mechanism of the phenomenon must be another here. Probably local adaptation plays a certain rôle by indirect vision (vide p. 72). (Conf. the fact that the fusion frequency of flicker by indirect vision decreases steadily with increasing exposure time, while by central vision the same function is fairly independent of the exposure time (*Granit*, 1936 [66])).

B. Experiments on the Influence of the Retinal Illumination.

During the experiments on the influence of the retinal illumination on *Troxler*'s phenomenon the visibility of a luminous point on quite unilluminated ground was registered by central vision. Light adaptation and artificial pupil was used as by the other experiments. The luminous point was produced by means of a square aperture, 30" by 30", placed in front of the usual illuminating system.

In order to maintain fixation during the periods when the point was invisible a red, luminous point, 100" by 100", of somewhat higher intensity had been placed on either side of the original point at a distance of 10'. By pressing down a telegraph key the subject indicated when the luminous point vanished and reappeared. The telegraph key closed and interrupted the current to an electro-magnetic writing device which wrote on a chronograph drum on which the time was

also marked. Readings were taken at various light intensities, they were always repeated in the opposite order to eliminate effects of fatigue. Each reading lasted abt. 45 seconds. The experiments were carried out with two subjects.

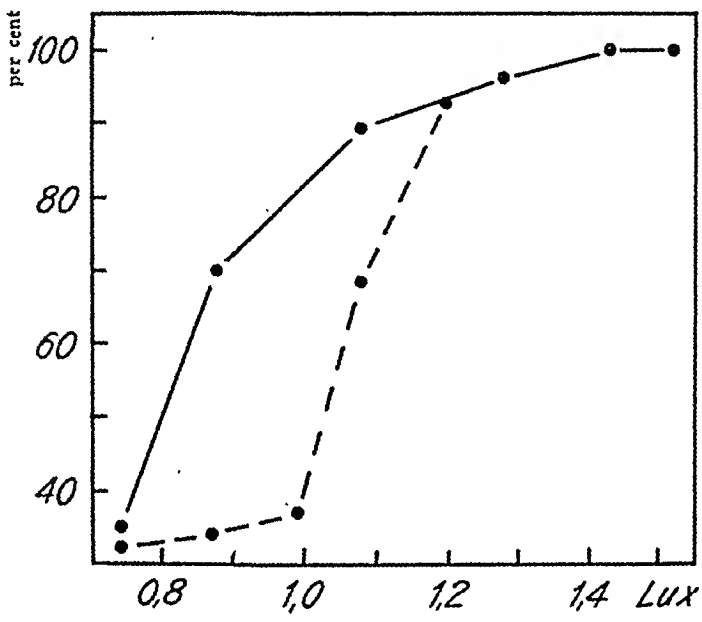


Fig. 35.

Troxler's phenomenon at varying retinal illumination.
Object: Luminous point (square, 30″ by 30″) on unilluminated ground.
Abscissa: Retinal illumination in lux.
Ordinate: The part of the time in per cent in which the point is visible.
———— Subject A.
– – – – Subject V.

By these experiments *Troxler*'s phenomenon (vide fig. 35) was shown to be clearly dependent on the retinal illumination, which substantiates the probability of the theory advanced and tells against the opinion that the movements of the eye is of special importance for the phenomenon, especially for the reappearance of the points.

According to this it should be possible to explain *Troxler*'s phenomenon for luminous points by central vision in the following manner: By low retinal illumination only a part of the retinal functional units are in their active

period, the others are refractory. When the *diffraction disc which constitutes the retinal* image of the point, during an elementary fixation (vide p. 56) falls on a refractory unit, the point will be invisible, but it will reappear if, during another elementary fixation, the image falls on an active, responsive unit.

CONCLUSIVE REMARKS ON CENTRAL AND INDIRECT VISION

A collected representation of the results of the investigation, with a view to the experimental results recorded in the previous chapters, is a task which naturally falls in two parts. In the first place an account shall be given of the relation between central vision and retinal structure, and secondly the points at which indirect vision differs from central vision at varying eccentricities shall be dealt with.

Central Vision.

It has been possible on essential points to confirm the theoretical analysis of the physiological components of the visual sense (vide chapter I).

The physiological component of the visual sense is divided in light sense, colour sense and the physiological resolving power.

Light sense is measured by the differential threshold (p. 6) which e. g. may be determined by means of the function »discrimination of thickness of line«, when the object is above a certain minimum length, so that the lines may be recognized as such, and one component of the complex function, the form sense, be eliminated thereby (p. 45).

Through experiments on the discrimination of thickness of line the differential threshold is found to be practically independent of the retinal illumination, until glare occurs and causes an increase (p. 173). With regard to the dimensions of the objects the width of the line — but not the length — has an influence on the discrimination factor in per cent within the field examined, i. e. the length of

the boundary line between the two »photometer fields« (p. 175).

Not only the discrimination of thickness of line, but also the minimum separabile may be a function of the intensity discrimination, if the contrast between the object and its background is not luminous/unilluminated or vice versa, but illuminated/less illuminated (pp. 24 and 144). The case may be the same for luminous squares on unilluminated ground, if the squares are so large that the min separabile becomes smaller than the resolving power for the optical system of the eye. (p. 138).

The colour sense shal not be dealt with here.

The physiological resolving power is measurer by means of the min. separabile for luminous squares of threshold brightness on unilluminated ground or unilluminated squares on luminous ground also of threshold brightness. The perception of the movement of a luminous point on unilluminated ground can also be used as an expression of the physiological resolving power (p. 150), as can the form sense under certain conditions and with certain reservations (p. 152).

By the experiments on the physiological resolving power it proved possible to reproduce the chief points of *Berger's* (1936, 1939) and *Berger & Buchthal's* (1938 a and b) experiments.

The size of the functional units in the retinal centre varies. The smallest units probably consist in single cones. The diameter (abt. 40" to 45" or 3.2 μ to 3.7 μ) may be determined as the min. separabile for unilluminated squares (the length of side of which is more than abt. 5') on luminous ground of threshold brightness (*Berger,* 1939 and p. 143). The average diameter of the functional units is abt. 3', and they must consequently consist of a group of cones (*Berger & Buchthal,* 1938 a and p. 107). The width of such a group may be determined in three ways. In the first place it may be determined as the min. separabile for luminous squares (length of side < abt. 40") of threshold brightness on unilluminated ground (p. 134). In the second place the width of the functional units may be measured by the smallest dis-

cernable movement of a luminous point (of threshold brightness) on unilluminated ground + the diameter of the diffraction disc + the excursion of the eye due to its constant oscillating movements (p. 148). Finally the width can be determined approximately as slightly less than half of the length of side which a luminous square of threshold brightness must have in order for its angularity to be recognizable (p. 152).

The functional units have an alternating activity, a certain number of them only being active at low retinal illumination, while the others are refractory. When the retinal illumination increases, a larger number of units are stimulated above their intensity threshold, whereby the number of active units per unit of area increases, and the form sense is improved correspondingly. (*Berger & Buchthal* 1938 b and p. 156). The alternating activity of the units may be observed directly by experiments on *Troxler's* phenomenon, which is most pronounced at low retinal illumination (p. 181).

In contradistinction to the functions mentioned hitherto the aligning power is always a complex function, as it is a function of the physiological resolving power as well as of the intensity discrimination. Thus the displacement threshold decreases with the length of the slit, while the discrimination of thickness of line is independent of the latter length (pp. 164 and 174).

Indirect Vision.

The light sense decreases very slowly with increasing retinal eccentricity, the intensity discrimination, measured by the discrimination of thickness of line at an eccentricity of 5°, being only half of what it is centrally. The dependence is slight compared with the experimental error. It has not been possible to prove the existence of the »physiological fovea« found by *Adler & Meyer* (1935). (p. 167).

The colour sense by indirect vision is not dealt with.

The physiological resolving power yields information with regard to the retinal structure and is therefore of the greatest interest when comparing central and indirect vision.

Indirect vision begins already at a distance of less than 2.7' from the centre of the visual field, the min. separabile for luminous points being smaller already at this eccentricity than it is centrally. Thus the extent of central vision on the retina is the same as that of the fixation area. (p. 108).

The physiological resolving power decreases rapidly with the eccentricity irrespective of the structural limits in the retina (the border of the fovea and the rod-free area). The diameter of the functional units increases with the eccentricity up to 3° in the horizontal and 2° in the vertical meridian, outside this boundary there are no functionally separated units, the nerve impulse probably spreading through the horizontal neural connections of the retina (pp. 139 and 150). The variation of the size of the functional units within the fovea is caused by variations in the average diameter of the cones, while outside the fovea both the distance between the individual cones and the greater number of cones per optic nerve fibre influences the size of the functional units. The dispersion of the impulse in the retina has the effect that the influence of the retinal eccentricity on the perception of movement is not so pronounced as it is on the min. separabile. (pp. 69 and 150).

The fact that from 2° and outwards the min. separabile in the vertical meridian differs from that in the horizontal must be caused by the distribution of the nerve fibres to the various parts of the retina. (p. 119).

The great importance of the attention for indirect vision appears from the fact that it is impossible to pay equal attention to all parts of a central visual field corresponding to more than a part of the fovea, and that a diversion of the attention from central to indirect vision or vice versa reduces the visual acuity centrally or peripherally respectively (p. 122).

The complex function, the aligning power, varies

somewhat with the retinal eccentricity, as the displacement threshold (p. 161) increases. The dependence is not so pronounced as that of the min. separabile, but more marked than that of the discrimination of thickness of line, and this is in conformity with the conception of the aligning power as a function of the light sense as well as of the physiological resolving power.

SUMMARY

Our knowledge of indirect vision is rather deficient. Even if it can be taken as settled that the visual acuity decreases with increasing retinal eccentricity, there are great discrepancies between the statements made by various authors with regard to the extent of the retinal area to which central vision is attached as well as to the rapidity with which the visual acuity decreases. Neither do the authors agree about the explanation of the difference between central and indirect vision. Some authors attach the greatest importance to the optical conditions, others consider structural factors and others again psychological factors as being of essential importance.

It was consequently thought desirable to carry out new experiments.

In order to find methods and objects suitable for comparative experimental investigations of central and indirect vision an analysis of the visual sense was carried out in the first chapter of this book; on the basis of the literature on this subject, the visual sense being for this purpose divided in light sense, colour sense and physiological resolving power.

The finer analysis consists in a division of the visual sense in »partial functions«, i.e. simple functions, as for instance the min. separabile and the differential threshold, by means of which special aspects of the visual function may be examined. Particular stress is laid upon such functions as may be expected to be suitable for measuring the physiological resolving power and thereby be correlated to the retinal structure.

The light sense is measured by the discrimination factor. The dependence of the differential threshold on light intensity, adaptation level and the size of the object is discussed, those investigations being left out of regard in which the adaptation level and light intensity have not been varied independently of each other. The intensity threshold is mentioned. It is emphasized that the smallest visible retinal image of a luminous point is obtained when the light intensity of the point is just above the intensity threshold.

In order to keep the extent of the present investigation within

certain limits the physiology of the colour sense is not dealt with in details, but a few of the results of the electro-physiological investigations of the last few years are mentioned shortly.

Under the heading, the physiologieal resolving power, an account is given of the possibilities of exaet localization of image points in the monoeular visual field, also of the importanee of the optical factors, especially the resolving power of the optieal system of the eye, and of the anatomieal strueture of the fovea.

The coneeption, »a functional unit«, is defined as the number of eones whieh through eonducting neurones are eonnected to the same optic ganglion cell. From our knowledge of the structure of the retina it may be eoneluded that the functional units in the centre of the fovea consist of at least one eone, while the possibility that the units are larger eannot be rejeeted on beforehand. As the size of the eones outside the eentre proper increases, the functional units must also vary in size (area) within the fovea.

The minimum separabile is defined, and its applicability as a measure of the physiological resolving power is discussed. An account is given of a theory advaneed by *Berger & Buchthal* according to whieh the min.separabile is only a function of the average size of the functional units and consequently of the physiological resolving power, when it is determined for luminous points of threshold brightness on unilluminated ground, and that the min.separabile for objects illuminated from before is always a funetion of the intensity diserimination (light sense). The theory is substantiated and expounded by a detailed review of previous investigations on the dependence of the min.separabile on the size of the object, its relation to the retinal mosaic and the influence of the light intensity. Under the latter heading it is mentioned how the intensity discrimination, the dispersion of light in the eye and the variation of the number of active funetional units per unit of area influences the min.separabile, when using different objects and conditions of illumination.

Other partial functions whieh must be considered to be functions of the physiological resolving power are then dealt with, namely the perception of the movement (of a luminous point) and the form sense (the ability to recognize the simple eontours of objects), their relationship to the retinal mosaic being outlined.

In eontradistinetion to the »partial functions« complex functions are taken to indieate functions the physiologieal basis of which is so complex that they so to speak »originates« from several partial functions. Of complex funetions the following are dealt with: the aligning power, the discrimination of thickness of line and the minimum legibile, which are all frequently considered to be, and used as, a measure of the visual aeuity. For each function its correlation to the other complex functions is further substantiated.

The second chapter, which deals with indirect vision, is divided in three sections. In the first section a description is given of how the indirect vision must differ from central vision on account of differences in the optical imagery and the structure of the retina and in the influence of attention and practice. A review of previous physiological investigations on indirect vision is given in the second section, and the third contains a discussion of the theoretical explanation of the differences between central an indirect vision.

It is explained why the field of central vision on the retina must be either equal to or larger than the retinal fixation area. From objective measurements of the movements during fixation as well as from subjective experiments it may be concluded that the fixation area must be between 2.5' and 5' in diameter, and the extent of central vision must either be of the same size or be still larger.

In the section about previous comparative investigations on central and indirect vision the papers in question are dealt with according to the division of the visual sense stated in chapter I, in order hereby to obtain a view of the problems of indirect vision and a basis for new experiments. It appears from the review of previous investigations that none of them have been carried out under such conditions, that it would be possible without essential reservations to correlate the results to the retinal mosaic.

In the SECOND PART a description is given of the author's own investigations consisting in comparative, sense-physiological experiments on central and indirect vision. Experiments on the following functions are recorded: the minimum separabile, perception of movement, form sense, aligning power and discrimination of thickness of line. In the following a summary of the main points of the experimental results shall be given.

The experiments confirm the correctness of the correlation of the individual functions examined which is given in chapter I. The discrimination of thickness of line must thus be interpreted as a function of the intensity discriminaton (light sense), when the object exceeds a certain minimum size. The min.separabile for objects illuminated from before may also serve as a measure of the light sense.

The physiological resolving power is measured by the min.separabile for luminous squares of threshold brightness on unilluminated ground or unilluminated squares on luminous ground also of threshold brightness. The perception of the movement of a luminous point on unilluminated ground can also be used as a measure of the physiological resolving power, as can the form sense with certain reservations and under certain conditions.

It proved possible by the experiments on the min.separabile and the form sense to reproduce the main points of *Berger's* and *Berger*

& Buchthal's investigations. The size of the functional units in the centre of the retina varies from single cones to larger groups of cones connected to the same optic nerve fibre. The average diameter of the functional units is abt. 3'. The functional units must be assumed to have an alternating activity, a certain number only being active at low retinal illuminations while the others are relatively refractory. When the retinal illumination increases a larger number of units will become stimulated above their intensity threshold, whereby the number of active units per unit of area increases. It seems possible to observe the alternating activity of the units directly through experiments on *Troxler's* phenomenon, the latter being most pronounced at low retinal illuminations.

In contradistinction to the functions hitherto mentioned the aligning power must always be of a complex nature, it being a function of the physiological resolving power as well as of the intensity discrimination.

The light sense by INDIRECT VISION, measured by the discrimination of thickness of line, decreases only slowly with increasing retinal eccentricity.

The physiological resolving power yields information on the retinal structure, and it is therefore of the greatest interest for comparisons between central and indirect vision. Indirect vision begins already at a distance which is less than 2.7' from the centre of the visual field, as the min. separabile even at this small eccentricity is larger than it is centrally. The extent of central vision on the retina is thus the same as the fixation area. The physiological resolving power decreases rapidly with the eccentricity, irrespective of the structural limits in the retina (the border of the fovea and the rod-free area). The size of the functional units increases with the eccentricity up to abt. 3° in the horizontal and 2° in the vertical meridian, outside which eccentricities the units are no longer functionally separated, and the nerve impulse must be assumed to spread through the horizontal neural connections of the retina. The variation of the size of the functional units is caused within the fovea by variations in the average diameter of the cones and outside the fovea by variations in the distance between the individual cones as well as by the larger number of cones connected to each optic nerve fibre. The dispersion of the impulse in the retina has the effect that the perception of movement is not influenced by the retinal eccentricity to such an extent as the min. separabile. The fact that outside eccentricities of 2° the values of the min. separabile in the vertical meridian differ from those in the horizontal meridian can probably be explained by the distribution of nerve fibres to the various parts of the retina.

That the attention is of great importance to indirect vision

appears from the fact that it is impossible to pay equal attention to all parts of a central visual field corresponding to more than a part of the fovea, and that a diversion of the attention from central to indirect vision or vice versa reduces the visual acuity centrally and peripherally respectively.

The complex function, *the aligning power*, varies somewhat with the retinal eccentricity, the displacement threshold diminishing with increasing eccentricity. This dependence is less marked than that of the min. separabile, but more pronounced than that of the discrimination of thickness of line, and seems to be in conformity with the conception of the aligning power as a function of the light sense as well as of the physiological resolving power.

Danish Summary

RÉSUMÉ

Vort Kendskab til det indirekte Syn er ret mangelfuldt. Selvom det kan betragtes som fastslaaet, at Synsstyrken aftager med stigende Nethindeexcentricitet, foreligger der modstridende Angivelser, dels om Størrelsen af det Nethindeomraade, der har direkte Syn, dels om hvor brat Synsstyrken aftager. Endvidere er der ikke Enighed om, hvorledes Forskellen imellem det centrale og indirekte Syn skal forklares. Nogle lægger Hovedvægten paa de optiske Forhold, andre betragter strukturelle og atter andre psykologiske Faktorer som Aarsag.

Ud fra disse Forudsætninger skønnes nye Forsøg ønskelige.

For at finde Metoder og Objekter, der egner sig til sammenlignende, eksperimentelle Undersøgelser over direkte og indirekte Syn, foretages paa Grundlag af den foreliggende Literatur i Afhandlingens første Kapitel en Analyse af Synssansen. Denne inddeles i Lyssans, Farvesans og den fysiologiske Opløsningsevne.

Den finere Analyse bestaar i en Opdeling af Synssansen i »Partialfunktioner«, d. v. s. simple Funktioner som f. Eks. minimum separabile og Forskelstærsklen, hvormed specielle Sider af Synssansen kan undersøges. Der lægges ved Gennemgangen af Funktionerne særlig Vægt paa saadanne, der kan forventes anvendelige som Maal for den fysiologiske Opløsningsevne og derved sættes i Relation til Nethindestrukturen.

Lyssansen maales ved Forskelsfølsomheden. Forskelstærsklens Afhængighed af Lysintensitet, Adaptationstilstand og Objektets Storrelse gennemgaas, idet de Arbejder udskydes, i hvilke Adaptationstilstand og Lysintensitet ikke er varieret uafhængigt af hinanden. Paavirkningstærsklen omtales. Det pointeres, at det mindste, synlige Nethindebillede af et lysende Punkt opnaaes, dersom dets Lysstyrke indstilles paa en Værdi lige over Paavirkningstærsklen.

Af Hensyn til Arbejdets Begrænsning ses bort fra en nojere Gennemgang af Farvesansens Fysiologi, idet dog nogle Resultater af de senere Aars elektrofysiologiske Undersøgelser paa dette Omraade kortfattet nævnes.

Under Omtalen af den fysiologiske Opløsningsevne

gøres først Rede for Betingelserne for nøjagtig Lokalisation af Billedpunkter i det monoculære Synsfelt, dels dq. optiske Forholds Betydning, specielt Opløsningsevnen for Øjets optiske System, dels den anatomiske Opbygning af fovea.

Begrebet »en funktionel Enhed« defineres som det Antal Tappe, der ved Ledningsforbindelser er knyttet til samme Opticusganglieeelle. Ud fra Kendskabet til Nethindestrukturen maa de funktionelle Enheder i foveas Centrum mindst bestaa af een Tap, medens Muligheden af større Enheder ikke paa Forhaand kan afvises. Da Tappene uden for selve Centrum tiltager i Størrelse, maa ogsaa de funktionelle Enheder variere i Størrelse (Areal) inden for fovea.

Minimum separabile defineres og dets Anvendelighed som Maal for den fysiologiske Opløsningsevne diskuteres. Der gøres Rede for en Teori hidrørende fra *Berger & Buchthal*, hvorefter min. separabile kun er et Udtryk for de funktionelle Enheders gennemsnitlige Størrelse og dermed for den fysiologiske Opløsningsevne, naar det bestemmes for lysende Punkter af Tærskelklarhed paa lysfri Grund, og at min. separabile for forfra belyste Objekter altid er en Funktion af Forskelsfølsomheden (Lyssansen). Teorien underbygges og uddybes ved en detailleret Gennemgang af tidligere Undersøgelser over min. separabiles Afhængighed af Objektstørrelsen, dets Forhold til Nethindemosaiken, samt Lysintensitetens Indflydelse. Under Omtalen af denne beskrives, hvorledes henholdsvis Forskelsfølsomheden, Lysspredningen i Øjet, samt en Ændring i Antallet af aktive, funktionelle Enheder pr. Arealenhed kan influere paa min. separabile ved forskellige Objekter og Belysningsforhold.

Af andre Partialfunktioner, der maa betragtes som Udtryk for den fysiologiske Opløsningsevne, gennemgaas derefter Opfattelsen af (et lysende Punkts) Bevægelse og Formsansen (Evnen til at erkende Genstandes primitive Konturer), idet deres Forhold til Nethindemosaiken skitseres.

I Modsætning til »Partialfunktionerne« forstaas ved sammensatte Funktioner saadanne, hvis fysiologiske Grundlag er saa sammensat, at de saa at sige »nedstammer« fra flere forskellige Partialfunktioner. Af sammensatte Funktioner omtales Noniussynsstyrken, Bedømmelse af Stregtykkelse samt Bogstavsynsstyrken, som alle tre hyppigt betragtes og anvendes som Maal for Synsstyrken. For hver enkelt Funktion begrundes dens Placering blandt de komplekse Funktioner nærmere.

Afhandlingens andet Kapitel, der handler om det indirekte Syn, falder i tre Afsnit. Først skildres, hvorledes det indirekte Syn maa afvige fra det eentrale paa Grund af Forskelligheder i den optiske Billeddannelse og den strukturelle Opbygning af Nethinden samt Opmærksomhedens og Øvelsens Betydning. Derefter refereres de tidligere fysiologiske Arbejder over det indirekte Syn og endelig

diskuteres de teoretiske Forklaringer af Forskellen mellem det centrale og det indirekte Syns Egenskaber.

Det begrundes, at det direkte Syns Omraade paa Nethinden enten maa være lig med eller større end Fiksationsomraadet. Saavel ved objektive Maalinger af Fiksationsbevægelserne som af subjektive Forsøg kan det sluttes, at Fiksationsomraadet maa være imellem 2,5' og 5' i Diameter og det direkte Syns Omraade enten have samme Størrelse eller være endnu større.

I Afsnittet om de tidligere sammenlignende Undersøgelser over direkte og indirekte Syn behandles de paagældende Arbejder i Overensstemmelse med den Inddeling af Synssansen, der er angivet i første Kapitel for at give et Overblik over det indirekte Syns Problemer og danne et Udgangspunkt for nye Forsøg. Af Gennemgangen fremgaar, at ingen af de hidtidige Undersøgelser er udført under saadanne Betingelser, at Resultatet uden væsentlige Forbehold vil kunne sættes i Relation til Nethindemosaiken.

I ANDEN DEL gøres Rede for egne Undersøgelser, bestaaende i sammenlignende, sansefysiologiske Forsøg over direkte og indirekte Syn. Der meddeles Forsøg over følgende Funktioner: Minimum separabile, Opfattelse af Bevægelse, Formsans, Noniussynsstyrke og Bedømmelse af Stregtykkelse. I det følgende skal samlet refereres Hovedpunkterne i Forsøgsresultaterne.

Forsøgene bekræfter Rigtigheden af den i første Kapitel angivne indbyrdes Placering af de undersøgte Funktioner. Bedømmelse af Stregtykkelse maa saaledes opfattes som en Funktion af Forskelsfølsomheden (Lyssansen), naar Objektet overskrider en vis Minimalstørrelse. Som et andet Udtryk for Lyssansen kan tjene min. separabile for forfra belyste Objekter.

Den fysiologiske Opløsningsevne maales ved Hjælp af min. separabile for lysende Kvadrater af Tærskelklarhed paa lysfri Grund eller lysfri Kvadrater paa lysende Grund, ligeledes af Tærskelklarhed. Ogsaa Opfattelsen af et lysende Punkts Bevægelse paa lysfri Grund kan benyttes som Udtryk for den fysiologiske Opløsningsevne, ligesom Formsansen med Forbehold og under visse Forsøgsbetingelser.

Ved Forsøgene over min. separabile og Formsansen viser Hovedpunkterne i *Bergers* og *Bergør & Buchthal's* Arbejder sig at kunne reproduceres. De funktionelle Enheder i Nethindens Centrum varierer indbyrdes i Størrelse fra enkelte Tappe til større Grupper af Tappe knyttet til samme Synsnervetraad. Den gennemsnitlige Diameter af de funktionelle Enheder er ca. 3'. De funktionelle Enheder maa antages at besidde alternerende Aktivitet, idet ved lav Nethindebelysning kun et vist Antal er aktive, de øvrige relativt refraktære. Naar Nethindebelysningen tiltager, bliver et større Antal Enheder paavirkede over deres Paavirkningstærskel, hvorved Antallet af aktive En-

heder pr. Arealenhed stiger. Enhedernes skiftende Aktivitet synes direkte at kunne iagttages ved Forsøg over *Troxler*'s Fænomen, der er mest udtalt ved lav Nethindebelysning.

I Modsætning til de hidtil nævnte Funktioner maa Noniussynsstyrken altid være kompleksk sammensat, dels et Udtryk for den fysiologiske Oplosningsevne, dels for Forskelsfølsomheden.

Lyssansen ved INDIREKTE SYN maalt ved Bedømmelse af Stregtykkelse aftager kun langsomt med tiltagende Nethindeexcentricitet.

Den fysiologiske Oplosningsevne giver Oplysninger om Nethindestrukturen og frembyder derfor størst Interesse ved Sammenligningen mellem direkte og indirekte Syn. Det indirekte Syn begynder allerede $<$ 2,7' fra Synsfeltets Centrum, idet selv ved denne ringe Excentricitet min. separabile for lysende Punkter er større end centralt. Det direkte Syn har saaledes samme Udbredning paa Nethinden som Fiksationsomraadet. Den fysiologiske Oplosningsevne aftager stærkt med Excentriciteten uden Indflydelse af de strukturelle Grænser i Nethinden (foveas og det stavfri Omraades Begrænsning). De funktionelle Enheder tiltager i Storrelse med Excentriciteten indtil ca. 3° i den vandrette og 2° i den lodrette Meridian, uden for hvilken Grænse, der ikke findes indbyrdes afgrænsede funktionelle Enheder, idet Nerveimpulsen maa antages at sprede sig gennem de horizontale Ledningsforbindelser i Nethinden. Variationen i de funktionelle Enheders Storrelse er inden for foveas Omraade foraarsaget af Ændringer i den gennemsnitlige Tapdiameter, uden for fovea har dels Tappenes indbyrdes Afstand, dels det større Antal Tappe pr. Synsnervetraad Betydning. Spredningen af Impulsen i Nethinden gor, at Opfattelsen af Bevægelse ikke paavirkes saa meget af Nethindeexcentricíteten som min. separabile. Naar min. separabile fra 2° og udefter er forskellig i den lodrette og vandrette Meridian, antages det at ligge i Nervetraadenes Fordeling til de enkelte Nethindepartier.

At Opmærksomheden har stor Betydning for det indirekte Syn, fremgaar af, at det ikke er muligt at knytte samme Opmærksomhed til et centralt Synsfeltomraade storre end svarende til en Del af fovea, og at en Spredning af Opmærksomheden fra det direkte til det indirekte Syn eller omvendt nedsætter Synsstyrken henholdsvis centralt og perifert.

Den sammensatte Funktion Noniussynsstyrken varierer noget med Nethindeexcentricíteten, idet Forskydningstærskien aftager udefter. Denne Afhængighed er mindre end min. separabiles og større end ved Bedømmelse af Stregtykkelse og synes at stemme med, at Noniussynsstyrken baade er en Funktion af Lyssansen og den fysiologiske Oplosningsevne.

REFERENCES

The bracketed figures [ ] refer to the pages in the text on which the authors are quoted.

Adler, F. H.: Problems in the Physiology of Visual Acuity. Arch. Ophth. *11:* 6, 1934 [4, 40, 45].

Adler, F. H. & M. Fliegelmann: Influence of Fixation on the Visual Acuity, ibid. *12:* 475, 1934 [56, 57].

Adler, F. H. & G. P. Meyer: The Mechanism of the Fovea. Tr. Am. Ophth. Soc. 71 meet. 1935 [44, 66, 83, 108, 166, 167, 169, 172, 185].

Adrian, E. D.: The Basis of Sensation, Lond. 1928, Pp. 122, p. 18, 106 [72, 130].

Adrian, E. D. & R. Matthews: The Action of Light on the Eye.

I. The Discharge of Impulses in the Optic Nerve .... J. Physiol. *63:* 378, 1927 a [72, 73, 74, 129].

II. The Processes Involved in Retinal Excitation, ibid. *64:* 279, 1927 b [74].

III. The Interaction of Retinal Neurones, ibid. *65:* 273, 1928 [73].

Anthonisen, H: Einige Untersuchungen üb. d. Beziehung zw. Sehschärfe u. Beleuchtung. Acta ophth. *14:* 437, 1936 [28].

Asher, L.: Über das Grenzgebiet des Licht- und Raumsinnes. Ztschr. f. Biol. *35:* 394, 1897 [9].

Aubert, H.: Physiologie der Netzhaut, Breslau 1865, Pp. 394, p. 7, 44, 55, 77, 103, 210, 228, 230, 233 [4, 7, 21, 26, 29, 46, 98, 105, 144, 177]).

Aubert, H.: Die Bewegungsempfindung. Arch. f. d. ges. Physiol. *39:* 347, 1886 [69].

Aubert, H. & Foerster: Raumsinn der Retina. Arch. f. Ophth. *3, 2:* 1, 1857 [62, 66, 68, 115].

Bartley, S. H.: Some Observations on the Organization of the Retinal Response. Am. J. Physiol. *120:* 184, 1937 [34, 35].

Bartley, S. H.: Some Factors in Brightness Discrimination. Psychol. Rev. *46:* 337, 1939 [34, 35].

Basler: Über das Sehen von Bewegungen. Arch. f. d. ges. Physiol. *115:* 82, 1906 [39].

Basler: Über das Sehen von Bewegungen, ibid. *124:* 313, 1908 [37, 39, 64].

Berger, C.: Untersuch. zur Methodik von Bestimmungen der Untersehiedsempfindlichkeit .... Skandin. Arch. f. Physiol. *71:* 173, 1935 [104].

Berger, C.: Weitere Untersuchungen üb. d. Unterschiedsempfindlichkeit (Auflösungsvermögen) .... ibid. *74:* 27, 1936 [21, 23, 80, 92, 103, 144, 184].

Berger, C.: Der kleinste Sehwinkel bei Verwendung lichtloser Quadrate auf selbstleuchtendem Grund .... ibid. *83:* 39, 1939 [23, 29, 32, 36, 49, 80, 96, 133, 137, 141, 147, 184].

Berger, C. & O. Boje: Über den Einfluss von Sauerstoffmangel auf das Auflösungsvermögen .... ibid. *77:* 129, 1937 [25].

Berger C. & F. Buchthal: Der Einfluss von Beleuchtung und Ausdehnung des gereizten Netzhautareals .... auf das Auflösungsvermögen .... ibid. *78:* 197, 1938 a [21, 23, 29, 32, 35, 38, 41, 78, 80, 89, 90, 92, 106, 123, 125, 126, 132, 133, 137, 144, 147, 184].

Berger C. & F. Buchthal: Formwahrnehmung und Funktion der Fovea, ibid. *79:* 15, 1938 b [34, 35, 40, 41, 82, 151, 156, 185].

Birch, Th.: The History of the Royal Society of London vol. III, London 1757, Pp. 520, p. 120 [20].

Bjerrum, J.: Undersogelser over Formsans og Lyssans. Disp. Kbh. 1882, Pp. 149, p. 1, 3, 105 [21, 40, 60, 78, 169].

Bjerrum, J.: Om Undersogelse af Synsfeltet. Medicinsk Selskabs Forhandl. Kobenhavn, *1888—89:* 58 [60].

Bloom, S. & S. Garten: Vergl. Unters. d. Sehschärfe .... Arch. f. d. ges. Physiol. *72:* 372, 1898 [67].

Bohr, N.: Licht und Leben. Naturwissenschaften. *21:* 245, 1933 [12].

Bois-Reymond, C. du: Scheinheit und kleinster Sehwinkel. Arch. f. Ophth. *32, 2:* 1, 1886 [21, 105].

Borchardt, H.: Beitr. z. Kenntnis der absoluten Schwellenempfindlichkeit .... Ztschr. f. Sinnesphysiol. *48:* 176, 1914 [12].

Bouma, P. J.: Beleuchtung und Verdunkelung. Philips techn. Rundschau. *4:* 25, 1939 [12].

Broca, A.: Causes rétiniennes de variation de l'acuité visuelle en lumiere blanche. J. de Physiol. et de Path. gen., *3:* 384, 1901 [33].

Broca, A. & D. Sulzer: La sensation lumineuse en fonction du temps, ibid. *4:* 632, 1902 [11, 73, 129].

Burchardt, M.: Internationale Sehproben. Kassel 1882, Pp. 43, p. 16 [61].

Chievitz, J. H.: Untersuch. üb. d. area centralis retinae. Arch. Anat. Physiol. Anat. Abt. Lpz. *1889.* Suppl. Bd. 139 [17, 54].

Craik, K. J. W.: The Effect of Adaptation on Differential Bright-

ness Discrimination. J. Physiol. *92*: 406, 1938 [8, 9, 10, 104].

Dieter W.: Über d. *Purkinje* Phänomen im stäbchenfreien Bezirk der Netzhaut. Arch. f. Ophth. *113*: 141, 1924 [108].

Dittler, R.: Die Physiologie des optischen Raumsinnes. In: *Schieck u. Brückner* (editt.) Kurzes Handbuch der Ophth. Bd: II, Berlin 1932, Pp. 1079, p. 382 [21].

Dobrowolsky, W. & A. Gaine: Ueber die Sehschärfe an der Peripherie der Netzhaut. Arch. f. d. ges. Physiol. *12*: 411, 1876 [40, 55].

Dobrowolsky, W. & A. Gaine: Ueber die Lichtempfindlichkeit auf der Peripherie der Netzhaut, ibid. *12*: 432, 1876 [60, 169].

Dohlman: Physikalische und physiologische Studien zur Theorie des kalorischen Nystagmus. Acta oto-laryng. Suppl. V, Upsala 1925, Pp. 196, p. 113, 119 [56, 57].

Dor, H.: Beiträge zur Electrotherapie der Augenkrankheiten. Arch. f. Ophth. *19*, 3: 316, 1873 [67].

Duke-Elder, W. S.: Textbook of Ophthalmology, vol. I, London 1932. Pp. 1124, p. 884 [4].

Duke-Elder, W. S.: Textbook of Ophthalmology, vol. II, London 1938. Pp. 2094, p. 903, 1194 [40, 178].

Einthoven, W.: Über die Beobachtung und Abbildung dünner Fäden. Arch. f. d. ges. Physiol. *191*: 60, 1921 [21].

Exner, S.: Bemerkungen über intermittierende Netzhautreizung, ibid. 3: 214, 1870 [60].

Exner, S.: Über das Sehen von Bewegungen. Sitz. Ber. Akad. Wiss. Wien, Math. nat. Kl. 72, 3: 156, 1875 [69].

Fazakas, A.: Über die zentrale und periphere Farbensehschärfe. Arch. f. Ophth. *120*: 555, 1928 [67].

Fechner, G. Th.: Ueber ein psychophysiches Gesetz und dessen Beziehung zur Schätzung der Sterngrössen. Abh. sächs. Ges. Wiss. Math. Phys. Kl. 6: 455, 1859 [7].

Ferree, C. E. & G. Rand: Interpretation of Refractive Conditions in the Peripheral Field of Vision. Arch. Ophth. *9*: 1925, 1933 [51].

Ferree, C. E., G. Rand & C. Hardy: Refraction for the Peripheral Field of Vision, ibid. 5: 717, 1931 [51, 52].

Fleischl, E. v.: Die Vertheilung der Sehnervenfasern über die Zapfen der menschlichen Netzhaut. Sitz. Ber. Akad. Wiss., Wien, Math. nat. Kl. 87: 246, 1883 [69].

Friedrich & Schreiber: Das Sehen des menschlichen Auges im Ultraviolett. Arch. f. d. ges. Physiol. *246*: 621, 1943 [13].

Gellhorn, E.: The Effect of O_2-lack, Variations in the CO_2-content of the Inspired Air, and Hyperpnoea on Visual Intensity Discrimination. Am. J. Physiol. *115*: 679, 1936 [25].

Gertz, H.: Ein Versuch über das direkte Sehen. Skandin. Arch. f. Physiol. *20:* 357, 1908 [56, 58].

Giraud-Teulon: Acuité visuelle, de ses éléments et de leur mesure. Ann. d'ocul. *81:* 215, 1879 [19, 21].

Graham, C. H.: The Relation of Nerve Response and Retinal Potential to Number of Sense Cells Illuminated in an Eye Lacking Lateral Connections. J. Cellul. & Comp. Physiol. *2:* 295, 1932 [74].

Graham, C. H., R. H. Brown & F. A. Mole jr.: The Relation of Size of Stimulus and Intensity in the Human Eye. I. Intensity Thresholds for White Light. J. of Exper. Psychol. *24:* 555, 1939 [12].

Graham, C. H. & C. Cook: Visual Acuity as a Function of Intensity and Exposure-time. Am. J. Psychol. *49:* 654, 1937 [127].

Graham, C. H. & N. Goldman: Intensity and Number of Cones in Foveal Stimulation. ibid.*44:* 275, 1932 [12].

Graham, C. H. & R. Granit: Comparative Studies on the Peripheral and Central Retina. VI. Inhibition, Summation and Synchronization of Impulses in the Retina. Am. J. Physiol. *98:* 664, 1931 [18, 36, 75])

Granit, R.: The Components of the Retinal Action Potential in Mammals and their Relation to the Discharge in the Optic Nerve. J. Physiol. *77:* 207, 1933 [72].

Granit, R.: Die Elektrophysiologie der Netzhaut und des Sehnerven. Acta ophth. Suppl. VIII, Kbh. 1936. Pp. 98, p. 3, 54, 66 [11, 18, 74, 180].

Granit, R.: Rotation of Activity and Spontaneous Rhythms in the Retina. Acta physiol. scand. *1:* 370, 1941 a [34].

Granit, R.: Isolation of Colour-sensitive Elements in a Mammalian Retina. ibid. *2:* 93, 1941 b [13, 53].

Granit, R.: Den principiella lösningen av färgproblemet. Nord. med. *11:* 2371, 1941 c [13].

Granit, R.: A Physiological Theory of Colour Perception. Nature Lond. *151:* 11, 1943 [13].

Granit, R. & P. Harper: Comparative Studies on the Peripheral and Central Retina II. Synaptic Reactions in the Eye. Am. J. Physiol. *95:* 211, 1930 [74].

Granit, R. & A. Munsterhjelm: The Electrical Response of Dark-adapted Frogs' Eyes to Monochromatic Stimuli. J. Physiol. *88:* 436, 1937 [34].

Granit, R. & P. O. Therman: Excitation and Inhibition in the Retina and in the Optic Nerve. J. Physiol. *83:* 359, 1935 [34].

Groenouw: Über die Sehschärfe der Netzhautperipherie und eine neue Untersuchungsmethode derselben. Arch. f. Augenh. *26:* 85, 1893 [60, 62].

Guillery, H.: Weitere Untersuchungen üb. d. Lichtsinn. Ztschr. f. Sinnesphysiol. *13:* 187, 1897 a [9, 60].

Guillery, H.: Über die Empfindungskreise der Netzhaut. Arch. f. d. ges. Physiol. *68:* 120, 1897 b [62].

Guillery, H.: Messende Untersuch. üb. d. Formensinn. ibid. *75:* 466, 1899 [41, 152].

Guillery, H.: Weitere Untersuch. zur Physiologie des Formensinnes. Arch. f. Augenh. *51:* 209, 1905 [47].

Guillery, H.: Sehschärfe. In: Handb. d. normal. u. pathol. Physiol. Editt. *Bethe, A. et al.* vol. XII, 2, Berlin 1931. Pp. 1641, p. 751, 763 [22, 42, 78, 132].

Gullstrand, A.: Die monochromatischen Aberrationen des Auges. In: *Helmholtz, H. v.:* Handb. d. physiologischen Optik III. Aufl. Bd. I, Hamburg 1909, Pp. 376, p. 375 [15].

Hartline, H. K.: Intensity and Duration in the Excitation of Single Photoreceptor Units. J. Cell & Comp. Physiol. *5:* 229, 1934 [13].

Hartline, H. K.: The Response of Single Optic Nerve Fibres of the Vertebrate Eye to Illumination. Am. J. Physiol. *121:* 400, 1938 [73].

Hartline, H. K.: The receptive field of optic nerve fibres. Am. J. Physiol. *130:* 690, 1940 [70].

Hartline, H. K. & C. H. Graham: The Spectral Sensitivity of Single Visual Sense Cells, ibid. *109:* 49, 1934 [13].

Hartline, H. K., H. Keffer & C. H. Graham: Nerve impulses from Single Receptors in the Eye. J. Cellul. & Comp. Physiol. *1:* 277, 1932 [6].

Hartridge, H.: Chromatic Aberration and Resolving Power of the Eye J. Physiol. *52:* 175, 1918 [15, 21].

Hartridge, H.: Visual Acuity and the Resolving Power of the Eye, ibid. *57:* 52, 1922 [21, 43].

Hartridge, H.: Vision. In: *Starlings* Principles of Human Physiology, edit. by *Ewans, C. Lowatt* 7. edit. London 1936. Pp. 1096, p. 381 [21, 45].

Hecht, S.: The Visual Discrimination of Intensity and the *Weber-Fechner* Law. J. gen. Physiol. *7:* 235, 1925 [7, 26, 33].

Hecht, S.: The Relation between Visual Acuity and Illumination, ibid. *11:* 255, 1928 [26, 33].

Hecht, S.: Eine Grundlage für die Beziehung zwischen Sehschärfe und Beleuchtung. Naturwissenschaften *1930, I:* 233 [33].

Hecht, S.: Die physikalische Chemie und die Physiologie des Sehaktes. Ergebn. d. Physiol. *32:* 243, 1931 [33].

Hecht, S.: A Theory of Visual Intensity Discrimination. J. gen. Physiol. *18:* 767, 1935 [7, 33].

Hecht, S.: La nature de la discrimination d'intensité dans la vision et la photoréception. J. de phychol. norm. & path. *33:* 161, 1936 [33].

Hecht, S. & E. U. Mintz: The Visibility of Single Lines at Various Illuminations .... ibid. 22: 593, 1939 [29, 124, 125].

Hecht, S. & G. Wald: The Visual Acuity and Intensity Discrimination of Drosophila, ibid. *17:* 517, 1934 [30, 33]

Hecht, S. & E. Wolf: The Visual Acuity of the Honey Bee, ibid. *12:* 727, 1929 [33, 34].

Heine, L.: Funktionsprüfung. In: *Axenfeld, Th.:* Lehrb. d. Augenh. 4. Aufl. Jena 1915, Pp. 800, p. 105 [21, 45].

Heinz, M. & F. Lippay: Üb. die Beziehungen zw. d. Unterschiedsempfindlichkeit u. d. Zahl d. erregten Sinneselemente. I Abhängigkeit d. Unterschiedsempfindlichkeit f. Helligkeiten v. d. Grösse des Netzhautbildes. Versuche b. foveater Beobachtung. Arch. f. d. ges. Physiol. *218:* 437, 1928 [9, 10, 59].

Helmholtz, H. v.: Handb. d. physiologischen Optik I. Aufl. Lpz. 1867. Pp. 874, p. 364 [177].

Helmholtz, H. v.: ibid. 2. Aufl. Hamburg, 1896. Pp. 971, p. 36, 259, 264, 425 [1, 21, 26, 69, 70].

Henius, K.: Die Abhängigkeit der Lichtempfindlichkeit v. d. Flächengrösse des Reizobjektes .... Ztschr. f. Sinnesphysiol. *43:* 99, 1909 [12].

Hensen, V.: Über das Sehen in der fovea centralis. Virchows Arch. *39:* 475, 1897 [178].

Hering, E.: Über die Grenzen der Sehschärfe. Ber. sächs. Ges. Wiss. Math.-Phys. Kl. *51, 3:* 16, 1899 [21, 42].

Hering, E.: Grundzüge der Lehre vom Lichtsinn. In: Handb. d. gesamten Augenheilk., begr. v. *v. Graefe u. Saemisch.* 2. Aufl. Bd. III, Kap. XII, Berlin 1925, pp. 294, p. 14, 141, 157 [8, 27, 31].

Hess, C.: Ueber einheitliche Bestimmung und Bezeichnung der Sehsehärfe. Arch. f. Augenh. *63:* 239, 1909 [46, 47].

Heuven, J. A. van: Über die Schschärfe bei übermässiger Beleuchtung. Acta ophth. *15:* 109, 1937 [21, 29, 105].

Hirschberg, J.: Ueber graphische Darstellung der Netzhautfunction. Arch. Anat. Physiol. Physiol. Abt. Lpz. *1878:* 324 [1, 115].

Hofmann, F. B.: Physiologische Optik. In: Handb. d. ges. Augenh. begr. v. *v. Graefe u. Saemisch.* 2. Aufl. Bd. III, Kap. XIII, Berlin 1925, Pp. 667, p. 19, 27, 28, 32, 34, 42, 55, 57, 58, 67, 70 [21, 22, 27, 40, 42, 46, 65, 69, 70, 78, 132, 144, 178].

Holm, E.: Central and Excentric Fixation. Acta ophth. *1:* 49, 1923 [55, 119].

Holth, S.: Det normale Synsorgans indirekte Stirreblindhed og dens Betydning under Synsfeltundersogelse. Norsk mag. f.

lægevidensk. 4. Række, *11:* Supp. Juli, 1896, Pp. 148, p. 110, 124 [177, 179].

Houston, R. A. & J. F. Shearer: The Quantum and Vision. Nature. Lond. *1930, II:* 437 [12].

Hueck, A.: Von den Grenzen des Sehvermögens. Arch. Anat. Physiol. Lpz. *1840:* 82 [60, 62].

Klein, F.: Das Eigenlicht der Netzhaut .... ibid. Physiol. Abt. *1911:* 191 [178].

Klenowa, E.: Sehschärfe beim Unterscheiden weisser Objekte auf schwarzem Grund und ihre Abhängigkeit v. d. Beleuchtungsintensität. Arch. f. Ophth. *133:* 402, 1935 [26, 28, 30].

König, A. & E. Brodhun: Experimentelle Untersuch. üb. d. psychophysische Fundamentalformel in Bezug auf den Gesichtssinn. Sitz. Ber. preuss. Akad. Wiss. *8, 1:* 917, 1888 [7].

König, A. & W. Uhthoff: Üb. die Beziehung zw. d. Sehschärfe u. d. Beleuchtungsintensität. Verh. phys. Ges., Berl. *4:* 79, 1886 [26, 28].

Königshöfer, O.: Das Distinctionsvermögen der peripheren Theile der Netzhaut. Disp. Erlangen 1876, Pp. 32, p. 12, 25 [62, 67].

Kolmer, W.: Die Netzhaut. In: Handb. d. mikr. Anat. des Menschen. Edit. *Möllendorf, W. v.*, Bd. III, 2, Berlin 1936, Pp.782, 394 [17].

Koster, W.: Untersuch. zur Lehre v. Farbensinn. Arch. f. Ophth. *41, 4:* 1, 1895 [53, 106].

Kravkov, S. W.: Sehschärfe u. Beleuchtung beim Unterscheiden weisser Objekte auf schwarzem Grunde. ibid. *131:* 452, 1933 [30, 32].

Kravkov, S. W.: Illumination and Visual Acuity. Acta ophth. *16:* 385, 1938 [26, 28, 30].

Kreiker, A.: Die psychische Komponente in der Sehschärfe. Arch. f. Ophth. *111:* 128, 1923 [48].

Kries, J. v.: Allgemeine Sinnesphysiologie, Lpz. 1923, Pp. 299, p. 144, 174 [104].

Kühl, A.: Die Reduktion v. Fernrohrbeobachtungen wegen Kontrastfehlers. In: *Seeliger, Hugo v.:* Probleme d. Astronomie, Berlin 1924, Pp. 475, p. 376 [16].

Kühl, A.: Die Abhängigkeit der Unterschiedsschwelle v. d. Objektgrösse u. Umfeldleuchtdichte. Ztschr. Instr. Kunde *60:* 293, 1940 [12].

Landolt, E. & Nuel: Versuch einer Bestimmung des Knotenpunktes für excentrisch in das Auge fallende Lichtstrahlen. Arch. f. Ophth. *19, 3:* 301, 1873 [51, 67].

Lazareff, P.: Studien üb. d. *Weber Fechnersche* Gesetz. Einfluss der Grösse des Gesichtfeldes auf den Schwellenwert der Gesichtsempfindung. Arch. f. d. ges. Physiol. *142:* 235, 1911 [9].

Lehmann, A.: Elemente der Psychodynamik. Lpz. 1905, Pp. 514, p. 183 [11, 73, 129].

Lythgoe, R. J.: The Mechanism of Dark Adaptation. Brit. J. Ophth. *24:* 21, 1940 [28].

Marx E. & W. Trendelenburg: Über die Genauigkeit der Einstellung des Auges beim Fixieren. Ztschr. f. Sinnesphysiol. *45:* 87, 1911 [57].

Mayer, T.: Experimenta circa visus aciem. Comment. soc. reg. scient. Gottingensis. *4:* 97, 1754 [9, 26].

Musylev, F. I.: Die Abhängigkeit der Sehschärfe v. d. Entfernung des Objektes. Acta ophth. *15:* 216, 1937 [62].

Nordenson, J. W.: Ögats undersökning. In: Nordisk lärobok i oftalmiatrik. Editt. *Ask & Berg.* Stockholm 1936, Pp. 533, p. 23 [45].

Nordenson, J. W.: Om synsskärpebestämningens internationalisering. Nord. med. *13:* 350, 1942 [47, 49, 50].

Öhrwall: Ögats rörelser under fixering. Upsala läkaref. förh. Ny Följd *17:* 441, 1912 [56, 57].

Østerberg, G.: Topography of the Layer of Rods and Cones in the Human Retina. Diss. Copenhagen 1935, Pp. 102, p. 18, 26, 64, 76, 84, 88 [16, 17, 53, 106, 107, 109, 110, 116].

Olmsled, J. M. D.: The Eye as an Optical Instrument. In: *Macleod's* Physiology in Modern Medicine. Ed.: *Bard, P.*, London 1938, Pp. 1051, p. 208 [45].

Pergens, E.: Ueber Factoren, welche das Erkennen von Sehproben beeinflussen. Arch. f. Augenh. *43:* 144, 1901 [47].

Pergens, E.: Analyse der *Landolt*'schen C-Figur zur Messung der Sehschärfe. Klin. Monatsbl. f. Augenh. *40, 2:* 311, 1902 [47].

Pergens, E.: Untersuchungen über das Sehen. Ztschr. f. Augenh. *9:* 256, 1903 [21].

Pergens, E.: Recherches sur l'acuité visuelle. Ann. d'Ocul. *135:* 11, 1906 [20].

Pickard, R.: The Light Threshold at the Macular and Perimacular Region. Tr. ophth. Soc. U. Kingdom *56:* 219, 1936 [12].

Podestá, H. H. & W. Aeffner: Der Einfluss der Prüffeldgrösse auf die Schwellenwahrnehmung des Auges. Arch. f. d. ges. Physiol. *243:* 666, 1940 [9, 10].

Polyak, S.: Minute Structure of the Retina in Monkeys and in Apes. Arch. Ophth. *15:* 477, 1936 [18, 54, 71].

Purkinje, J.: Beobachtungen und Versuche zur Physiologie der Sinne I. Prag 1823, Pp. 176, p. 76, 78 [177].

Purkinje, J.: Beobachtungen und Versuche zur Physiologie der Sinne II. Berlin 1825, pp. 192, p. 4, 21, 22 [1, 54, 119].

Riccó, A.: Üb. d. Beziehungen zw. d. kleinsten Sehwinkel u. d. Lichtintensität. Zentralbl. f. Augenh. *1877:* 122 [9].

Roelofs, G. O. & L. Bierens de Haan: Über den Einfluss von Beleuchtung und Kontrast auf die Sehschärfe. Arch. f. Ophth. *107:* 151, 1922 [21].

Ronne, H.: Zur Theorie u. Technik der *Bjerrumschen* Gesichtfelduntersuchung. Arch. f. Augenh. *78:* 284, 1915 [60].

Ronne, H.: Øjensygdomme. Kbh. 1940, Pp. 125, p. 9 [45, 47].

Rubin, E.: Synsoplevede Figurer. Disp. Kbh. 1915. Pp. 228, p. 186, 191 [16, 44].

Ruppert: Ein Vergleich zw. dem Distinktionsvermögen u. d. Bewegungsempfindlichkeit der Netzhautperiferie. Ztschr. f. Sinnesphysiol. *42:* 409, 1908 [67, 69].

Russel, H. N.: The Minimum Radiation Visually Perceptibile. Astrophys. J. *45:* 60, 1917 [12].

Schadow, G.: Die Lichtempfindlichkeit der peripheren Netzhautteile .... Arch. f. d. ges. Physiol. *19:* 439, 1879 [67].

Schieck, F.: Netzhaut. In: *Henke & Lubarsch:* Handb. d. spec. path. Anat. u. Histologie, Bd. 11, 1, Berlin 1928. Pp. 1042, p. 580 [17].

Shlaer, S.: The Relation between Visual Acuity and Illumination. J. gen. Physiol. *21:* 165, 1937 [30].

Snellen, H. & E. Landolt: Die Funktionsprüfungen des Auges. In: Handb. d. ges. Augenh. Editt. *Graefe & Saemisch* 1. Ausg. Bd. 3, Lpz. 1874, Pp. 500, p. 3 [45].

Steinhardt, J.: Intensity Discrimination in the Human Eye. I The Relation of $\Delta I/I$ to Intensity. J. gen. Physiol. *20:* 185, 1936 [9].

Stiles, W. S. & B. H. Crawford: The Liminal Brightness Increment for White Light for Different Conditions of the Foveal and Parafoveal Retina. Proc. roy. soc. London B *116:* 55, 1934 [9].

Stratton, G. M.: A New Determination of the Minimum Visible and its Bearing on Localization and Binocular Depth. Psychol. Rev. *7:* 429, 1900 [37].

Stratton, G. M.: Visible Motion and the Space Threshold. ibid. *9:* 433, 1902 [39, 42].

Sulzer, D. E.: Fonction de la rétine. In: Encyclop. Franc. d'Opht. Editt. *Lagrange & Valude* vol. III, Paris 1904, Pp. 1134, p. 561 [21].

Sundberg, C. G.: Ub. d. Blickbewegung u. d. Bedeutung des indirekten Sehens f. d. Blicken. Skandin. Arch. f. Physiol. *35:* 1, 1918 [56, 57].

Troxler, I. P. V.: Ueber das Verschwinden gegebenen Gegenstände innerhalb unseres Gesichtskreises. Ophth. Bibl. Jena *2:* 7, 1804 (161) [177].

Tschermak, A.: Augenbewegungen. In: Handb. d. norm. u. path. Physiol. Editt. *Bethe et al.* Bd. *12, 2,* Berlin 1931, Pp. 1641, p. 1055 (56).

Tscherning, M.: Physiologic Optics, Philadelphia 1924, Pp. 425, p. 44 [58].

Volkmann, A. W.: Sehen. In: Handwörterb. d. Physiol. Edit. *Wagner, R.* Bd. 3, 1, Braunschweig 1846, Pp. 872, p. 334 [62, 67].

Volkmann, A. W.: Physiologische Undersuch. im Gebiete der Optik. Lpz. 1863, Pp. 268, p. 86 [26].

Wald, G.: Area and Visual Threshold. J. gen. Physiol. *21:* 269, 1938 [12].

Weber, E. H.: Der Tastsinn u. das Gemeingefühl. In: Handwörterb. d. Physiol. Edit. *Wagner, R.* Bd. 3, 2, Braunschweig 1846, Pp. 588, p. 526, 527, 531 [19, 51, 67].

Wertheim, T.: Ueber die Zahl der Scheinheiten im mittleren Theile der Netzhaut. Arch. f. Ophth. *33, 2:* 137, 1887 [21, 62, 68, 105, 108].

Wertheim, T.: Über die indirekte Sehschärfe. Ztschr. f. Sinnesphysiol. *7:* 172, 1894 [63, 115].

Weymouth, F. W. & E. E. Andersen: Visual Perception and the Retinal Mosaic. Am. J. Physiol. *64:* 561, 1923 [63].

Weymouth, Hines, Acres, Raff & Wheeler: Visual Acuity within the Area Centralis .... Am. J. Ophth. *11:* 947, 1928 [63, 108].

Wilbrand, H. & A. Saenger: Die Neurologie des Auges. Bd. 3, 1 Wiesbaden 1904, Pp. 474, p. 168 [17]

Wilcox, W. W.: The Basis of the Dependence of Visual Acuity on Illumination. Proc. nat. acad. sc. *18:* 47, 1932 [29, 32, 124, 126].

Wilcox, W. W.: An Interpretation of the Relation between Visual Acuity and Light Intensity. J. gen. Psychol. *15:* 405, 1936 [21, 32].

Wilcox, W. W. & D. Mc. L. Purdy: Visual Acuity and its Physiological Basis. Brit. J. Psychol. *23:* 233, 1933 [22, 33].

Wolffberg, L.: Analytische Studien an Buchstaben u. Zahlen .... Arch. f. Ophth. *77:* 409, 1910 [21].

Wright, W. D. & R. Granit: On the Correlation of some Sensory and Physiological Phenomena of Vision. Brit. J. Ophth. Supp. IX, Lond. 1938, Pp. 80, p. 67, 70 [27, 33].

Wülfing, E. A.: Ueber den kleinsten Gesichtswinkel. Ztschr. f. Biol. *29:* 199, 1892 [42].

Young, T.: Oevres ophtalmologiques de *Thomas Young* traduites par *M. Tscherning.* Copenhague 1894, Pp. 248, p. 144 [67].

ACTA PHYSIOLOGICA SCANDINAVICA
VOL. 9, SUPPLEMENTUM XXVIII

FROM THE PHARMACOLOGICAL DEPARTMENT OF THE
CAROLINE INSTITUTE, STOCKHOLM.

STUDIES ON THE HISTAMINOLYTIC POWER OF PLASMA WITH SPECIAL REFERENCE TO PREGNANCY

By

AXEL AHLMARK

Stockholm 1944

CONTENTS.

	Page
Introduction	5
Chapter I. Some characteristics of histaminase	7
Distribution of histaminase	8
Enzyme specificity	8
Method of preparation	8
The structure of the enzyme	9
The enzyme-substrate reaction	10
Chapter II. Critical analysis of earlier investigations on the histaminolytic power of the blood	16
Methods involving the determination of the undecomposed histamine	16
Methods involving the determination of the oxygen uptake	23
Methods involving the determination of ammonia formation	24
Methods involving the determination of the discoloration of indigo disulphonate	25
Own investigations.	
Chapter III. Determination of the histaminolytic power of plasma	27
The determination of histamine	27
The inactivation of histamine in plasma	38
Method for the determination of the histaminolytic power	43
Some characteristics of the histaminolytic principle	50
Chapter IV. The histaminolytic power of blood in animals	54
Guinea-pigs	54
Rats	56
Rabbits	57
Cattle and other animals	58
Chapter V. The histaminolytic power of plasma in man	60
A. Men and non-pregnant women	60
B. Pregnancy and puerperium	63
Healthy subjects	63
Imminent abortions, extra-uterine pregnancy	75
Albuminuria and toxaemia of pregnancy	81
Premature parturition, hydatiform mole	85

Page

Chapter VI. The histaminolytic power of placenta and its relation to that of plasma 87
Method of extraction 88
Measuring the effect of the histaminolytic power 89
Results: Rats 94
Other animals 95
Man 96
Chapter VII. Diagnostic and prognostic value of the histaminolytic power 99
Summary 103
References 106

Introduction.

Since BEST (1929) first proved the existence of an enzymatic inactivation of histamine in the organism, a considerable number of investigations have been carried out which have illustrated the more direct nature of this phenomenon from a chemical viewpoint. They have shown that one or several enzyme systems (histaminase, diamine oxidase) participate in the cleavage, and we also know about certain important properties in them as well as about the reaction of the destruction.

Only occasional observations have so far been made concerning the relation in the organism of the enzymatic histamine inactivation. ZELLER, BIRKHÄUSER, MISLIN & WENK (1939) have surveyed our knowledge as to the distribution of the enzyme in the various organs of humans and animals. As regards the enzyme in the human organism we know that it occurs first and foremost in kidney, adrenals and liver, but apart from this our knowledge is limited.

However, a number of investigations have shown that pregnant women have considerably higher histaminolytic power than is normally the case [MARCOU et. al. (1938), WERLE & EFFKEMANN (1940 a), ZELLER & BIRKHÄUSER (1940)]. In a preliminary report I (1944) have given a method to determine the histaminolytic power of blood, by means of which it was possible to show a considerable increase in the histaminolytic power during pregnancy from the seventh week after the last menstruation. The increase was so strong that it could be seen from one day to the next during the first half of the pregnancy. In a case of imminent abortion with a good prognosis the histaminolytic power increased almost normally, while in a case of extra-uterine pregnancy it gradually decreased, and this indicated that the relation of the histaminolytic power might be of assistance when prognosticating uterine bleedings.

In this paper I found it suitable first to give a résumé of the most important particulars with regard to our present knowledge

of the histaminolytic enzyme and a critical analysis of earlier investigations on the histaminolytic power of the blood. A detailed study has subsequently been devoted to the histaminolytic power of the blood in animals and man under different conditions.

As a first stage to illustrate the histaminolytic power of the blood I have worked out a quantitative method for its determination. Secondly by means of some introductory experiments I have tried to ascertain whether conditions observed in man concerning the enzymatic inactivation of histamine correspond to similar ones observed in certain animal species.

In order to give a picture of the quantitative relation of the histaminolytic power in man a number of tests have been carried out on several healthy men and women. As the main interest has been centred on pregnancy, special attention has been devoted to this state. Some investigations have also been carried out with a view to illustrating the nature of the histaminolytic power.

Earlier investigations indicate that in some cases of illness during pregnancy the histaminolytic power of the blood deviates from the normal, and this question has been taken up and illustrated by means of some cases of uterine bleedings, premature parturition, albuminuria and toxaemia of pregnancy as well as hydatiform mole.

It has also for a long time been known that placenta contains a strong histaminolytic enzyme, but its relation to the histaminolytic power of the blood has not been investigated. The author has therefore gone into the question as to whether there exists any such relation in man, rat and rabbit.

In order to illustrate the physiological relations of the histaminolytic power the author has finally analysed his results as to whether and to what extent the deviations from the normal indicate an abnormal state.

CHAPTER I.

Some characteristics of histaminase.

The oldest statements in literature about an enzyme able to destroy histamine originate from BEST (1929). He investigated the stability of the histamine in the lung tissues of cattle and horse, and found that both naturally occurring and added synthetic histamine disappears in the course of a few days in a suspension of lung tissue in saline incubated at 37° C. One year later BEST & MC HENRY (1930) published an extensive investigation of the histamine inactivating principle in organ extracts from dog, and they suggested that "the substance or system, which produces a change in structure responsible for the loss of physiological activity of histamine" should be called histaminase.

Subsequent to BEST & MCHENRY and the latter's collaboration with GAVIN (1932, 1935) several workers have been occupied with investigations concerning the physico-chemical properties of histaminase. The problem was taken up by EDLBACHER (1937), who in collaboration with ZELLER began to publish a series of reports on histaminase; this research work was later taken over by ZELLER, who either alone or with several other co-workers directed our attention in a large number of publications to the histamine-destroying enzyme in several respects. Lastly if we mention SWEDIN (1943 a, b, 1944), who has prepared the hitherto purest enzyme preparations and has thus been able to clear up many earlier vague and conflicting statements with regard to the effects of the enzyme, we shall have mentioned those whose works have marked the most important progress in our knowledge of the chemistry of histaminase. This chapter will give a concentrated description of the main features in this development; certain details which are of interest when studying the blood histaminase will be dealt with later.

Distribution of histaminase. As has already been stated the enzyme was discovered in the lung tissues of cattle and horse. Later investigations, however, have shown that in comparison with other organs the lung is pretty badly supplied with the enzyme in question; BEST & MCHENRY have already shown that the enzyme content in the kidney and intestine of dog was considerably greater than in the lung. MCHENRY & GAVIN (1935) showed inter alia the extremely high enzyme activity in pig and sheep kidneys and EDLBACHER & ZELLER demonstrated that the enzyme activity was mainly confined to the cortex. For further literature concerning the distribution of the enzyme, see ZELLER, BIRKHÄUSER, MISLIN & WENK (1939), ZELLER (1941 a) and others, which will be dealt with later.

Enzyme specificity. At first it was supposed that histaminase inactivated only histamine. ZELLER (1938 a) also showed that it could certainly be distinguished from monoamine oxidase and d-aminoacid oxidase, but he also showed that putrescine, cadaverine, agmatine as well as ethylenediamine cause increased oxygen consumption and ammonia formation, when added to a histaminase preparation made from kidney. This led to the conclusion that the enzyme destroyed these substances as well. In agreement with this observation ZELLER suggested that the enzyme should be called *diamine oxidase*. In another communication the same year ZELLER (1938 b) stated that the enzyme also acts upon trimethylenediamin, spermine and spermidine. ZELLER (1938 b, 1940 a, 1941 b) has shown that also other amines and diamines — besides those mentioned — are attacked by the enzyme or inhibit the substrate-enzyme reaction. Like histamine other substituted diamines are also attacked. — As will be dealt with later the destruction of histamine probably consists of two reactions, the breaking of the side chain and the rupture of the imidazole ring.

Method of preparation. BEST & MCHENRY prepared a stable histaminase powder by extracting beef kidney with acetone and ether. The activity in the powder was practically speaking the same as that of the kidney tissue from which it was prepared. Other preparations have been produced by EDLBACHER & ZELLER (1937), KIESE (1940) LASKOWSKI (1942), SWEDIN (1943 a, b, 1944) and STEPHENSON (1943). Even though it is not possible to explain in detail from literature statements the relative strength of the histaminase preparations, it is nevertheless clear that the

hitherto strongest preparations have been produced by STEPHENSON and SWEDIN, the purest one by SWEDIN. He extracted ground hog kidney with phosphate buffer, pH 7.7, and precipitated it with ammonium sulphate. After dialysis with physiological saline and water the solution was heated for 10 mins. up to 45—50° C. A slight precipitate was removed by centrifugation and the preparation was again precipitated with ammonium sulphate. After dialysis the author obtained a preparation with a 35 times stronger enzyme effect than the pig kidney. Finally by electrophoresis a further purification was obtained up to 124 times of the original material. The preparation thus obtained showed considerable lability. By treating with heat, however, the durability of the preparation considerably increased, and the author stated that he has been able to keep his preparations for months at — 10° C without any loss of activity.

The structure of the enzyme. ZELLER (1938 b) showed that both pepsin and trypsin completely inactivated the enzyme, and this along with the fact that it could be dialysed for weeks without losing its activity, confirmed that diamine oxidase is of a protein nature. Earlier investigations, however, had already been carried out giving certain information as to the character of the enzyme. Thus BEST & MCHENRY showed that potassium cyanide in 0.0005 molar concentration inhibited the histamine inactivating effect of kidney powder, and EDLBACHER & ZELLER, who had, moreover, found that the reaction continued unchecked in the presence of 40 % CO_2+60 % O_2, therefore considered that there was reason to believe that histaminase was of a hemin character. GEBAUER-FUELNEGG & ALT (1932) had, however, already made some observations difficult to reconcile with this theory, showing inter alia that the cyanide inhibition was not complete; 0.002 and 0.0002 molar concentration respectively thus inhibited the oxidation by only 60 % and 25 %. They showed, moreover, that neither 95 % CO + 5 % O_2 nor the presence of 0.1 molar sodium pyrophosphate in the reaction mixture inhibited the enzyme effect. When ZELLER et al. afterwards more closely analysed the effect of the cyanide ions on the histamine-histaminase reaction and found that in this case the cyanides had nothing to do with their known effect on metal catalyzers, they found reason to abandon their previous theory of the hemin character of the enzyme. They stated, however, that the reaction in the intact cell might possibly be catalyzed by a hemin enzyme. The authors go on to state, however,

that for a long time they have observed how the active enzyme preparation has been yellow and that the activity has been parallel with the degree of the colouring. By means of special treatment they have also been able to produce an enzyme showing positive lumi — flavine reaction. LASKOWSKI was able to isolate a fraction containing hemoglobin which was inactive in itself (0.5—0.8 saturation with ammonium sulphate), but which considerably increased the effect of the active fraction (0.2—0.5 saturation). When purifying the enzyme by means of electrophoresis as mentioned above, SWEDIN (1943 a, b) found that the active principle accompanying a fraction with a flavine protein character could be separated from an inactive complex containing hemin proteids. The inactive hemin protein fraction, however, showed no constant activating effect on the histaminase.

Even though the enzyme is not yet isolated, SWEDIN (1943 b) nevertheless considered that he was able to state that the diamine oxidase is probably a flavine protein. SWEDIN (1944) also gave an account of how he had succeeded in destroying the enzyme reversibly.

Several investigations indicate that a carbonyl group is included in the prosthetic group of the enzyme. After BLASCHKO (1938) informed us that guanidine inhibits the histamine — histaminase reaction, ZELLER (1938 b) analysed this matter more closely and was able to state that the inhibition was competitive, and that even other carbonyl reagents, such as hydroxylamine and semicarbazide, have an inhibitive effect. WERLE (1940 a) found that the phenylhydrazine and other ketone reagents gave strong inhibition.

On the basis of the condition just described that a quantity of carbonyl reagent causes a strong inhibition of the enzyme-substrate reaction, while a number of enzyme poisons, such as carbon monoxide, arsenic trioxide, pyrophosphate, urethane, sodium azide and sodium fluoride exercise no important inhibition, ZELLER et al. put forward the hypothesis that the cyanide ions exercise their effect as a carbonyl reagent. The cyanide ions combine with the aldehyde group of enzyme, thus preventing the enzyme-substrate reaction. The fact that the power of KCN to inhibit the decomposition of amines with different affinity to the enzyme varies, indicates this.

The enzyme-substrate reaction. The chemical course in the destruction of histamine and the other diamines in connection

with the effect of the diamine oxidase is not known in detail, but there are definite statements as regards certain links in the chain of reaction. In important respects, however, different authors put forward different investigation results, which as a rule must be attributed to the fact that different pure enzyme preparations have been used at these investigations. Most probably several systems of enzyme are active in the complete destruction of histamine, and this adds to the difficulty of judging relevant problems.

McHENRY & GAVIN determined the liberated quantity of ammonia and found that it corresponded to an ammonia formation from one of the three nitrogen atoms in the histamine. They found on the one hand very good agreement between the quantity of histamine that disappeared out of a histamine-histaminase mixture after different periods of time, and on the other the ammonia formed during the reaction. Thus they were able to show a formation of one molecule of ammonia for every molecule destroyed. By following the quantity of amino-nitrogen during the inactivation of the histamine, these authors were able to show in their later paper (1935) that the ammonia formed comes from the amine group of the side chain, and that no amino compounds are formed when the imidazole ring is broken.

The question as to which nitrogen molecule in the histamine was released at the time of the deamination was later gone into by EDLBACHER & ZELLER. These authors also followed the inactivation of the histamine by determining the ammonia formed. They assumed that the nitrogen atom in the ammonia originated from the imidazole nucleus, but ZELLER (1938 a) abandoned this view: since histaminase formed ammonia even when acting upon aliphatic diamines, he then adopted McHENRY's & GAVIN's view that it must be the nitrogen in the side chain of the histamine that causes the ammonia formation.

BEST & McHENRY found that an uptake of gas, probably oxygen takes place during the reaction. More thorough manometric studies of the oxidation of histamine were published by GEBAUER-FUELNEGG & ALT. They observed an oxygen consumption at the histamine — histaminase reaction, which was in a certain relation to the disappearance of the intestine activity of the histamine. The investigations of these authors, however, did not lead to any definite molar relation between the oxygen consumed and the quantity of histamine inactivated. ZELLER

(1938 a) found that during a first phase of the reaction approximately two oxygen atoms were consumed per molecule substrate. Simultaneously with the consumption of an atom of oxygen a molecule of ammonia was formed.

As has already been said, McHENRY & GAVIN had shown that the biological activity of the histamine had disappeared when it had formed a molecule NH_3 at the substrate-enzyme reaction. Consequently according to ZELLER's investigation, which has just been related, the biological activity in histamine has disappeared when an atom of oxygen has been taken up by the histamine-histaminase mixture. This circumstance was made the object of an investigation by ZELLER, SCHÄR & STAEHLIN (1939), but they were not able to verify it. When they put the oxygen uptake in relation to the quantity of histamine inactivated, they found that it takes a little more than one atom of oxygen per histamine molecule destroyed.

KIESE (1940) first made things clear. He produced two purified enzymes of different strengths from the pig kidney cortex; the enzyme obtained after the first fractionation with ammonium sulphate took up one molecule of oxygen per molecule of histamine, while an enzyme produced after repeated precipitatations only took up half a molecule of oxygen. The biological activity of the histamine had completely disappeared after half a molecule of oxygen had been taken up. Consequently it is obvious that the earlier contradictory results obtained must be referred to the defective purity of the enzyme preparations used. KIESE's results as regards the consumption of oxygen in the histamine-histaminase reaction have later been verified by LASKOWSKI (1942) and STEPHENSON (1943). SWEDIN (1943 a, b) has shown that when the reaction with a preparation purified by means of electrophoresis is followed in a WARBURG apparatus, the uptake of oxygen suddenly decreases when an atom is consumed. After that the consumption continues much less rapidly till one molecule of oxygen has been taken up per molecule of histamine. The biological activity has entirely disappeared when the first atom of oxygen has been consumed.

ZELLER (1938 a) also went into the question of other reaction products in the histamine-histaminase reaction. Thus he considered that he had indirectly shown that hydrogen peroxide forms intermediarily, and in support of this he refers to the fact that hemoglobin is converted into methemoglobin if added to a

histamine-histaminase mixture. Further, ZELLER showed that ethyl alcohol increases the consumption of oxygen in the reaction mixture, and in another communication (ZELLER 1938 b) he stated that indigo disulphonate decolorizes during the diamine-diamine oxidase reaction, which he attributes to the effect of the hydrogen peroxide formed. STEPHENSON also supported ZELLER's statement as to the H_2O_2 formation with the histamine-histaminase reaction, but he also admits that there is no direct proof of this, and this "is most readily explained by assuming that hydrogen peroxide, formed during the oxidative deamination of histamine, causes a secondary oxidation through the action of another enzyme such as peroxidase". The assumption as regards the H_2O_2 formation, however, was criticized by LASKOWSKI, who, by means of coupled experiments with alcohol, could show no increased consumption of oxygen as a sign of intermediary H_2O_2 formation. Furthermore, LASKOWSKI found that if catalase was added to a mixture of pig kidney histaminase purified by means of ammonium sulphate and acetone precipitates, and histamine, the oxygen consumption was not diminished which would have been the case, if hydrogen peroxide had formed intermediarily. Nor could SWEDIN (1944) observe any decrease in the oxygen consumption when crystalline horse liver catalase had been added to his purified histaminase preparation.

Thanks to SWEDIN's investigations, however, the positive results will also be able to be explained, for he found that crystalline peroxidase considerably increases the consumption of oxygen during the first phase of the histamine-histaminase reaction. If catalase was also added, moreover, the total consumption of oxygen decreased in the first phase of the reaction. SWEDIN considered that this was due to the fact that the united action of histaminase and peroxidase produce peroxide, which is destroyed by the catalase, when the liberated oxygen is consumed instead of the atmospheric. The hydrogen peroxide shown by ZELLER and STEPHENSON can thus be explained by their enzyme preparations being polluted by hemin proteids with peroxidase effect.

In this connection it should also be stated that LASKOWSKI obtained a marked increase in the oxygen consumption with the histamin-histaminase reaction when adding hemoglobin and hemin. STEPHENSON also stated that hemoglobin causes an increased oxygen uptake. SWEDIN, however, found no change in the histamine-histaminase reaction even when adding much more

crystalline horse hemoglobin than peroxidase. Thus, the results of the former authors may be due to an unspecific peroxidase reaction.

Before discussing other reaction products more closely, we shall refer to the investigations carried out with a view to clearing up the relation of the imidazole ring to the histamine-histaminase reaction. BEST & MCHENRY determined the changes in the reaction mixtures of the imidazole content and found that in certain cases they were roughly parallel with the loss of histamine. Their experiments, however, do not show that the rupture of the ring is absolutely essential to bring about the loss of the biological activity. STEPHENSON could find no change in the imidazole during the consumption of one atom of oxygen. He assumed that BEST's & MCHENRY's observation must be attributed to the effect of another tissue factor than histaminase. SWEDIN found that the rupture of the ring in his purified histaminase preparation took place in the very first stage of the reaction. If peroxidase were added, however, when the consumption of oxygen considerably increased, the breaking of the imidazole ring decreased to a very great extent, and the author assumed that the addition of peroxidase brings about a competitive relation between the two simultaneous reactions, the breaking of the side chain and the rupture of the imidazole ring. The addition of catalase had no effect on the rupture of the ring.

Of the destructive products in the histamine-histaminase reaction, ammonia has been the one most easy to determine and the first in which a stoichiometric relation to the substrate content destroyed has been shown. Our knowledge as to other destructive products is more defective. ZELLER, STERN & WENK (1939) found that aldehyde was formed by the oxidative deamination and STEPHENSON reported that one molecule of aldehyde is produced when one atom of oxygen is consumed during the deamination of putrescine. In the case of histamine the ratio of atoms of oxygen to molecules of aldehyde was slightly higher than 1, indicating an oxidation of the reaction product.

There is only scanty information in literature with regard to other products in the histamine-histaminase reaction. EDLBACHER & ZELLER stated that they could show a carbonyl compound with the formula $C_4H_4N_2O_4$ which crystallizes nicely as a dinitrophenyl hydrazone. After alkalizing and blowing air through a digested mixture of histamine and enzyme they have observed a black

precipitate resembling melanin which appeared to a lesser extent the purer the enzyme preparation used.

It is not known how the histamine destruction proceeds in detail. Everything, however, indicates that there is a simultaneous oxidative deamination of the side chain and a rupture of the imidazole ring. SWEDIN's (1944) investigations showed that by adding peroxidase it is possible to get the two reactions to proceed at different rates, and he also demonstrated that the presence of this enzyme is probably necessary to get the H_2O_2 composition described by several authors.

In this connection it seems only right that the question of the *nomenclature* of the enzyme should be gone into. As long as histamine was looked upon as being the only substance catalyzed by the enzyme it was called histaminase. After discovering that other diamines were also catalyzed, ZELLER changed and gave it the name of diamine oxidase. Since then MCHENRY and ZELLER (see ZELLER 1938 b) have agreed that the enzyme which breaks the ring shall be called histaminase in a narrow sense, and the enzyme which oxidatively deaminates the side chain shall be named diamine oxidase. As, however, the side chain is most likely attacked before the ring opens, the enzyme which breaks the ring will not affect the histamine as such but a destructive product of it, and therefore SWEDIN (1943 a) considered the name agreed upon inadequate. He called all the histamine destructive enzyme histaminase.

CHAPTER II.

Critical analysis of earlier investigations on the histaminolytic power of the blood.

Apart from an isolated investigation of the power of guinea-pig serum to detoxify histamine, which was carried out long before anything was known about the existence of any histamine destroying enzyme (BUSSON & KIRSCHBAUM, 1912), the development of the methods for determining the histamine inactivating power of the blood has largely synchronized with the development of our knowledge of histaminase. Thus we find that several of the methods employed in the study of the enzyme have been made use of when testing the blood, and we shall now examine some of them. As far as the blood is concerned, the biological determination of histamine has been employed to show the disappearance of the amine. When carrying out blood investigations, moreover, measurements have been made of the quantity of oxygen consumed and ammonia formed and also — as a sign of the presumed intermediary H_2O_2 formation — of the discolourment of the indigo disulphonate.

Methods involving the determination of the undecomposed histamine.

Even only a few years after histamine had been first synthesized, BUSSON & KIRSCHBAUM (1912) investigated, whether guinea-pig serum possessed any histamine destructive power. Serum, which had been mixed with 1 % histamine, was shaken for 4 hours, but the writers could observe no inactivation of the substance.

The next information concerning the histaminolytic power of the blood was given by BEST & MCHENRY (1930). Although they give no detailed account of their method, they seem to have

added histamine to defibrinated dogs blood, and after 4 hours' incubation at 37° C determined the remaining quantity of histamine on the blood pressure of the cat. They found that in 4 hours 600 ml dogs blood never inactivated more than 2 mg histamine base and in six tests of normal human blood, each of 50 ml, they could not show any histaminase activity. No quantitative statements are given as to the initial histamine percentage and time of incubation, nor as to the accuracy of the determinations, and therefore it cannot be said what histaminase effect may possibly have escaped their notice.

YEN & CHANG (1933) endeavoured to find a correlation between the tendencies of different animal species to anaphylactic shock and the histamine destructive power of the blood. Histamine in concentrations varying from ca 0.01 to ca. 0.5 mg histamine phosphate pr ml was added to blood. The tests were incubated for 24 hours at 37° C. The remaining quantity of histamine was determined on blood pressure on the atropinized cat. Thus tested guinea-pigs and human blood showed no histamine destructive effect, whereas blood from cats and dogs almost always did. No systematic investigations of the kinetics of the histamine inactivation were published, nor was it stated with what degree of accuracy the histamine determinations were carried out. It is therefore not possible to form any definite idea as to the magnitude of the histaminolytic effect in the negative tests.

MARCOU et al. (1938) and MARCOU (1938) examined the histaminolytic power of the blood in man and in some laboratory animals. 2 ml 1 % "Bayer 205" and 30 γ histamine phosphate were added to 5 or 10 ml blood, and then the mixture was incubated for $^1/_2$ hour at 37° C. After that the remaining quantity of histamine in the blood was determined according to BARSOUM's & GADDUM's (1935) method. The histaminolytic effect was expressed as the percentage of histamine destroyed during the incubation. The same relative inactivation of the histamine in varying initial quantities of histamine was found in double blood tests. No histamine destruction in blood from rabbits and cats could be observed with MARCOU's method, but in guinea-pig MARCOU discovered varying results, while determinations in blood from man and dog always showed such an effect. As regards man the writers stated that blood taken from pregnant women just before parturition shows this quality "to [illegible] igh degree, while venous blood in normal man displaye[illegible] same, although very much less".

MARCOU et al. are the first workers in this field to give a method for quantitative estimation of the histamine destructive effect. They gave only a few such data, however. As an example, they mentioned that human blood can show an inactivation of 20—30 % and the guinea-pigs blood 15 %. The writers made no statements as to the accuracy of the method adopted.

When making a supplementary test on account of MARCOU's investigation, it is true that I have been able to establish that human blood during $^1/_2$ hour's incubation destroys from 0—100 % of the histamine added. The statement that the relative inactivation of histamine is not due to the quantity of blood in the digestion mixture I have not been able to verify. Thus I have found about the same amount of inactivated histamine as well at the initial concentration of 1,5 γ amine per ml as at 0.6 γ per ml and about 30 % less at the concentration 30 γ per ml. In another case I found the absolute amount of inactivated histamine to be only about 10 % less at 42.5 γ per ml than at an initial concentration of 18 γ per ml.

A variant of MARCOU's determination method was published a year later by UNGAR & PARROT (1939). Histamine and physiological saline were added to 0.5 ml plasma, so that the histamine concentration was 0.2 γ/ml, after which the tests were incubated for 2 hours at 38°. The remaining quantity of histamine was determined on guinea-pig intestine and the histamine destructive effect was expressed by means of the percentage of inactivated histamine. With this method the writers found normally 0—20 % in dogs plasma and 10 % in a woman of 52 years.

ALBUS (1939) investigated the activity of histaminase in healthy and "latent allergic" subjects. The histaminase activity of the blood was expressed as the decrease in the effect on the blood pressure which took place when a blood preparation mixed with histamine was incubated. In healthy subjects ALBUS considered that he had established the existence of histaminase.

A systematic investigation of the histamine inactivation effect of the blood by means of a biological method was first undertaken by WERLE & EFFKEMANN (1940 a). As their method brought about interesting results, which are generally referred to, it will be more closely analysed in this paper. The test was undertaken in the following manner.

Venous blood was taken from subjects on empty stomachs and defibrinated by shaking with beads after which the blood was imme-

diately tested. Increasing quantities of histamine dihydrochloride from 3, 6, 9 etc. up to 50 γ were added to samples each of 3 ml blood, after which Tyrode's solution was added to make the volume 4 ml. The digestion solutions were then shaken for $1^1/_2$ hours at 37° C, and were afterwards cooled down to 0° C. During the incubation oxygen was bubbled through the test vessels. The remaining quantity of histamine was determined on isolated atropinized guinea-pig intestine. At first the blood sample was tested to which 3 γ histamine had been added; if no histamine was found in this, the sample with 6 γ was tested. If all the histamine were destroyed in this sample as well, then the 9 γ test was resorted to and so on, until the test was reached in which histamine was still to be found. The inactivating effect was given as the quantity of histamine dihydrochloride inactivated in the first test in the series, which did not show total inactivation. For example, if it was found in the sixth sample — to which 18 γ histamine had been added from the first — that 1.2 γ remained, the histaminase equivalent was given as 18 γ minus 1.2 γ i. e. 16.8. When examining blood from non-pregnant women it appeared that there was always histamine remaining in the first sample initially containing 3 γ histamine.

According to the above definition the writers found that the amount of histaminase in non-pregnant women was between 0.3 and 1.7 and as a rule 1.2, in pregnant women it was 5.0—46.8, and from this they concluded that the histaminase content of the blood increases 4—30 times during pregnancy.

The theoretical basis of this method of histaminase determination will now be examined, and the question will be particularly gone into as to whether and to what extent WERLE's & EFFKEMANN's histaminase equivalents allow of a definite numeral comparison between the histaminolytic power in the respective blood samples. These authors produce no detailed statement of possible sources of error nor do they give any report on the accuracy of the method.

One fact which seems to be of the greatest importance in this question is the manner in which the authors have carried out the determination of the remaining quantity of histamine. It has for a long time been known that blood contains substances, which in various ways affect the motility of the isolated gut. These substances render it impossible to make a direct determination of the histamine of a blood sample by comparing its intestinal activity with the effect of a known histamine solution as a standard. The sample must be first so prepared that its other pharmacologically active substances are excluded as far as possible. WERLE & EFFKEMANN, however, do not seem to have taken these facts into sufficient consideration. They have put into the saline in which

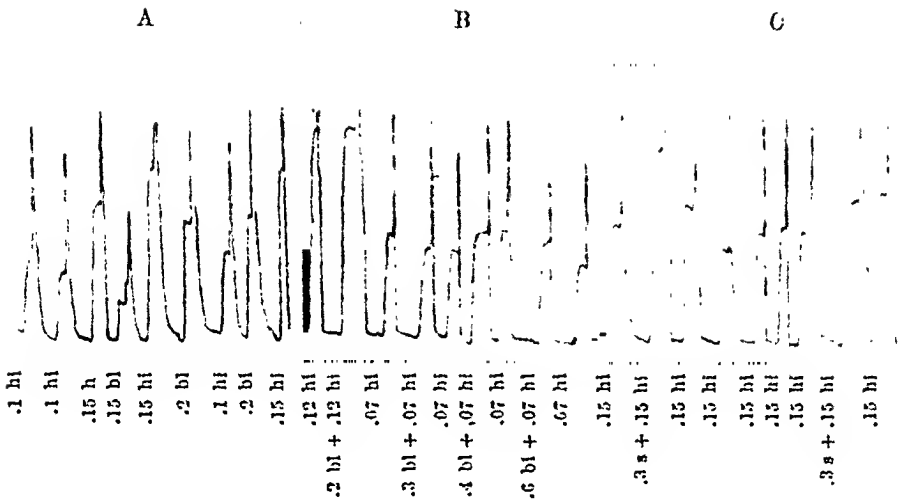


Fig. 1. Guinea-pig ileum in Tyrode's solution. Volume of bath 20 ml.

A. Tyrode's solution containing 0.5 γ atropine/ml. hi = γ histamine dihydrochloride. bl = ml defibrinated blood containing 1 γ histamine dihydrochloride/ml.

B. Tyrode's solution containing 0.5 γ adenylic acid/ml. hi = γ histamine dihydrochloride. bl = ml defibrinated blood.

C. Tyrode's solution containing 0.5 γ atropine/ml. hi = γ histamine dihydrochloride. s = ml serum from a pregnant woman.

For explanations, see text!

the intestinal preparation has been hung up such large quantities of blood, to which histamine has been added, that in many cases the quantity of blood seems to amount to more than 1/100 of the suspension. From my experiments it is clear that the reaction of the guinea-pig intestine to histamine decreases considerably even at very low blood concentrations. Thus fig. 1 A shows that even a blood percentage of 0.75 in the intestine bath diminishes the contraction by about one half. Experiments on another gut showed that the same percentage in the suspension fluid does not make the intestine react at all to 0.0045 γ histamine/ml (in this case it was possible to observe a distinct record when 0.0012 γ histamine/ml suspension fluid was added without any simultaneous supply of blood).

The determination of histamine in the blood preparation without consideration being taken to the above mentioned facts must thus lead to erroneous conclusions. This will make it obvious that the appreciation of WERLE's & EFFKEMANN's statement in favour of the activity of histaminase in defibrinated blood must be taken with the greatest reserve. As, however, the authors' results in other respects constitute valuable progress in

our knowledge of the histamine inactivating effect of the blood it seems worth while to analyse more closely the sources of error already mentioned in order to be able to draw from them conclusions concerning the other results of the authors.

For this purpose it would be of value to establish the relative inhibition of the contraction of the intestine that a certain blood concentration in the intestine bath causes. I have carried out experiments which, however, show that there is no simple relation between blood concentration and intestinal inhibition, it having been proved that the inhibition with one and the same blood concentration varies for various quantities of simultaneously added histamine. Thus, for example, 1 % of blood may bring about 68 % inhibition of the contraction that has been caused by 0.12 γ histamine, while the same blood percentage only gives 15 % inhibition of a 1.2 γ contraction on the same intestine preparation. Thus the determination of the histamine must in all circumstances be considered to be uncertain.

The reason for the inhibition observed is not explained, but it may be due to adenosine compounds of the blood, which possess an inhibitive effect on the non-striated muscles. This is indicated by an experiment carried out with Tyrode's solution mixed with 0.5 γ adenylic acid (Merck)/ml instead of with atropine. On this intestine preparation the blood added caused no strong inhibition of the histamine contractions (fig. 1 B) as was the case when the intestine was atropinized, which may indicate that the intestine contraction was already inhibited to a certain extent by the adenylic acid which had been added. However, I consider a full report on this question to be irrelevant to this paper.

WERLE & EFFKEMANN found, as has already been mentioned, that in blood from non-pregnant women, to which had been added 3 γ histamine dihydrochloride, it was usually possible to show only 1.8 γ after $1^1/_2$ hours' incubation. The difference observed they interpreted as an expression of the power of the blood to inactivate histamine. From the above analyses it also seems possible to explain the difference as an expression of the power of the blood to inhibit contraction. It is impossible, however, to determine without further investigations whether the whole or only part of this difference can be explained by this inhibition.

The inhibition of the contractions of the gut in the presence of human blood are nothing like so noticeable when serum is substituted for blood. In many cases, however, there is another drawback

with serum added to the intestine. The sensitivity of the intestine to histamine often undergoes considerable changes when serum is repeatedly added. Fig. 1 C illustrates this. When the intestine has for several minutes shown good sensitivity and histamine in serum is added, it is often found that the sensitivity increases or decreases, the gut thus being not fit for quantitative histamine determination.

The disturbances in the sensitivity of the intestine when estimating the histamine of pregnant serum are sometimes more, sometimes less pronounced. The result, however, with this method means very time wasting and tedious analytical work without arriving at anything but an approximate estimation of the histamine percentage of the serum sample. What has been said about serum will also largely apply to plasma.

In their investigations Warner & Ingraham (1936) have also measured the histamine destroying effect in plasma and serum. They found at 37° in both from a non-pregnant woman that was considerably less than in defibrinated blood. Thus they could never observe any inactivation in the samples mixed with 3 γ histamine dihydrochloride, whereas the γ samples in 4 cases out of eleven showed an inactivation, but in two cases an activation of 1–2 γ histamine.

The above mentioned critical viewpoints are not of the same significance when judging the results which Warner & Ingraham obtained with blood from pregnant women. In some cases they found such strong inactivation of histamine that it cannot solely be explained as a lowering in the sensitivity of the intestine to histamine. Thus when they for an investigation of 30 γ histamine dihydrochloride in a blood sample from a pregnant woman, only a fraction of this inactivation can be assigned to the effect of the inhibition of the intestine. The results of the plasma and serum analyses are still more interesting, for here the authors have found that the inactivation goes up to 17 in plasma and 7 in serum.

If it can be definitely said that the authors have proved the histamine inactivation in defibrinated blood, serum and plasma in pregnant women to exist, the question remains as to what extent the histaminase quantities stated can be considered sufficient for the accurate determination of the histaminolytic principle.

Abuse, Barsoum, Ingram & Amin (1941) added 3 γ histamine phosphate per ml serum and incubated the mixture for 30 minutes

at 37° C. Two separate determinations were made on each sample, the first immediately after the addition of histamine, the second after incubation. The difference expressed in percentage between the results of the two determinations represented the histaminolytic index.

Methods involving the determination of oxygen uptake.

The first to carry out investigations of the histaminolytic enzyme of the blood by means of a gasometric method were ZELLER, BIRKHÄUSER, MISLIN & WENK (1939). They found regular signs of the diamine oxidase effect in blood from man. In a later paper ZELLER & BIRKHÄUSER (1940) went more closely into details as to their method: 3 ml serum dialysed for 6—24 hours were put into a manometric vessel. A quantity of 0.3 ml n/10 cadaverine was added as a substrate and the test was incubated at 37° C. There was a control with buffer solution instead of cadaverine for every test with serum. The authors emphasise the importance of the dialysis: undialysed serum showing more than ten times as strong an oxygen uptake as the dialysed in the control tests.

In the control tests i. e. without the addition of any diamine, the serum from non-pregnant women usually showed an oxygen uptake of less than 2 c.mm., in one case, however, it went up to 6.5 c.mm. (time of incubation 8—21 hours). With the cadaverine addition there was generally a further oxygen uptake of some c. mm., but there were even cases when the oxygen consumption was greater in the control test than in the cadaverine one. According to these conditions the uptake of serum from pregnant women incubated between 2 and 19 hours was between 23.6 and 122.3 c. mm. O_2 or about 50 times more than that taken from non-pregnant.

With double determinations of the same serum to which had been added substrate, the authors' table 2 showed that they agree on an average as near as to within 2 to 3 c.mm. of each other. In cases with high absolute consumption this variation is of less importance, but where the consumption is little the variation must cause considerable uncertainty in the results. The authors did not publish any double determinations of the control tests, and as there is no reason to presume that they would show any really better agreement than those just mentioned, it must be assumed

that the difference in the oxygen uptake, which may be attributed to the effect of the substrate, is concomitant with an average mean error of ± 3 to 4 c.mm. With consideration to this, an oxygen uptake of less than 6 to 8 c.mm. confirmed by a single determination, will not definitely be thought to indicate the existence of diamine oxidase in serum.

The oxygen consumption observed in the serum tests from pregnant women seems to be beyond doubt and a manifestation of the existence of diamine oxidase. Such does not seem to be the case with non-pregnant subjects. The high oxygen consumption in the control tests obviously renders the results more difficult with the cadaverine tests. It is still more doubtful with the values from the tests in which the control test has a greater consumption of oxygen than that carried out with substrate. In their first mentioned paper, however, the authors interpreted these results as being the consequence of an inhibition of the diamine-diamine oxidase reaction, and they say inter alia that by means of a long dialysis they have been able to show an increased oxygen consumption after the addition of substrate. "Nur in einer kleinen Zahl von Fällen von Hemmung reichte dic Zeit nicht aus, um zu entscheiden, ob ein Abbau stattgefunden hatte, wie wir es für wahrscheinlich halten."

If the authors' tests with serum from non-pregnant women are summed up, there is nevertheless a tendency to an increase of the oxygen consumption when adding substrate. It has already been pointed out repeatedly, however, that there is usually a considerably greater uptake of oxygen in non-purified preparations than there is in purified ones by deamination of the substrate. In this respect it is probable that even the oxygen consumption in serum does not entirely correspond to a destruction of the substrate, and consequently the values given must only be considered a relative measure of the diamine oxidase content of the serum.

Methods involving the determination of ammonia formation.

The determination of the ammonia formed in serum during the histamine-histaminase reaction has been carried out by ZELLER (1940 b, 1941 c). Cadaverine was added to dialysed serum and the mixture incubated at 37° C for 4 to 16 hours, and the NH_3 formed

was determined. It was not always possible to show any NH_3 formation in serum from non-pregnant women, the NH_3 formation in these tests was often less than in the control test simultaneously carried out without substrate. In no case, however, did ZELLER observe any greater formation of nitrogen than 0.4 γ ammonia nitrogen per ml. serum per 16 hours' incubation. Serum from pregnant women showed a formation of 18 γ ammonia nitrogen under the same conditions.

Methods involving the determination of the discoloration of indigo disulphonate.

The discoloration of indigo disulphonate (IDS) in the diamine-diamine oxidase reaction shown by ZELLER (1938 b) has also been made use of when showing the histaminolytic enzyme in blood. ZELLER employed for the experiment 4 to 5 ml serum incubated with cadaverine and IDS for 24 hours. The discoloration did not take place with serum from non-pregnant subjects even after incubation for several days, but with serum from pregnant women a quantity of only 0.2 ml will cause discoloration. NEUMANN & EBBINGE (1942) found that the reaction was more easily read if instead of serum they used a solution of the protein fraction which precipitates at 0.33 saturation of ammonium sulphate. There was only one case out of 30 non-pregnant women in which IDS was discoloured. On the other hand the reaction was most often positive with pregnant subjects.

NEUMANN'S & EBBINGE'S investigations showed that the diamine oxidase in serum is precipitated almost quantitatively at 0.33 saturated ammonium sulphate solution. This statement is not in agreement with the results obtained by other authors, who endeavoured to purify the enzyme. Instead they have found the maximum activity in precipitates between about 0.4 and about 0.6 saturation with ammonium sulphate. It therefore seemed doubtful, as stated by NEUMANN & EBBINGE, whether there was any advantage worth mentioning by carrying out the test with only the fraction precipitating at 0.33 saturation. In some investigations of the distribution of the histamine inactivating factors on separate protein fractions I also found that there was a considerable quantity in the fractions precipitated at more than 0.33 saturation of ammonium sulphate.

The earlier investigations on the histaminolytic power of blood have been carried out under extremely varying conditions, consequently the results obtained are not commensurable. Fundamental objections in certain cases may also be raised to the isolated investigations. However, taken altogether, the investigations show that dogs blood inactivates histamine, whereas contradictory results are obtained from other animals. The same thing has also been noticed when adopting a biological technique as regards man. The scarcity of experimental data, however, has made it impossible to show the reasons for the various results arrived at by different authors. By means of chemical methods it has been considered possible to show the existence of histaminase in blood; but on closer examination the fact must not be excluded that the effect shown in some cases may be explained in another manner. With all the methods of investigation blood from pregnant women shows a decided increase in its histaminolytic power, and this subject will be dealt with later.

CHAPTER III.

Determination of the histaminolytic power of plasma.

From the critical analysis of earlier investigations it was clear that the chemical methods for proving the histaminolytic principle were combined with a relative large error when determining blood tests with low activity. Several authors have, however, by means of biological methods been able to establish a rather accurate determination of the histaminolytic power also in blood with low activity. It therefore seemed as though the biological method was more suitable than the chemical one when determining such blood tests.

I have chosen to investigate whether and to what extent it is possible to analyse plasma tests with low activity by means of a biological method with a satisfactory accuracy. In order to be able to compare blood tests with low and high histaminolytic power it is necessary to work under the same or similar conditions, and I have therefore also carried out investigations of tests with high activity by means of the same biological method.

The determination of histamine.

Two different methods can be used for the quantitative determination of histamine in biological material. Firstly, it can be determined *chemically*, secondly, *biologically*. The chemical way of determination is generally based on a formation of coloured compounds indicated by PAULY, which appear when the imidazole ring of the histamine combines with diazo compounds. The reaction is not specific, the same thing applying to e. g. histidine. It is not adopted when determining extremely small quantities of histamine. ($< 1\ \gamma$). The biological methods for the quantitative determination of histamine are based on its pronounced pharmacological effects on various organs, particularly on in-

testinal motility and blood pressure. Nor are these methods specific, however, numerous substances existing in biological material influenee the organ or system of organs tested and render the interpretation of the determination results difficult. As opposed to the ehemical some of the biological methods, however, have the advantage of being considerably more sensitive (> 0.02 γ histamine/ml). In this chapter a description is given of a biologieal method for determining histamine.[1]

Preparatory treatment of the blood samples.

As has already been said, when it is a question of showing biologieally very small quantities of histamine in blood, consideration must be taken to other substances existing in the blood, sueh as choline, adenosine eompounds, potassium and eertain, less well known substances which influence the biological preparation. Several methods have consequently been worked out for the chemical preparatory treatment of the blood, by which is aimed at a destruction or elimination of the disturbing substances, at the same time as the histamine is preserved to the greatest possible extent. By taking certain measures the biological substrate has also been made more sensitive to histamine than to the other substances.

When determining histamine in blood it is generally the method introdueed by BARSOUM & GADDUM (1935) that is adopted or a modification of it. These authors found that if blood, after being precipitated with trichloraeetic acid, whieh was then removed with ether, was boiled with hydrochloric aeid, the adenosine compounds and other substances were destroyed, and by dissolving the extracts evaporated to dryness with alcohol saturated with sodium chloride the potassium content in the preparation beeame negligible too. When testing the extracts of biologieal preparations particularly sensitive to choline, the authors recommended the addition of atropine, as this will abolish the effect of the cholinc. BARSOUM's & GADDUM's original method, later on modified in a couple of details by GADDUM (cit. CODE 1937), has been closely analysed by several authors. For instanee, CODE (1937) pointed out that the elimination of the trichloracetic acid with ether is unnecessary, since the acid gets destroyed in any case when it is afterwards boiled with the hydrochloric

[1] In this paper by histamine I mean histamine base.

acid. CODE also found a considerable loss of histamine during the alcohol extraction, and therefore he suggested replacing it with a water extraction. MAC INTOSH (1938) confirmed CODE's observation that there is a considerable loss of histamine when the alcohol extraction takes place.

ANREP et al. (1939) found that the histamine equivalent in blood obtained according to CODE was higher than that arrived at with BARSOUM's & GADDUM's method. ANREP et al. stated that the reason for this is a substance in the remainder of the water solution which affects the gut and which the alcohol is not able to dissolve. This substance is to be found in the red blood-corpuscles but not in serum. Later KWIATKOWSKI (1941) showed that it was potassium. According to CODE, however, potassium does not exist in such quantities in the extract that it interferes with the contractions of intestine to histamine.

Investigations showing that the substance determined in the preparation is histamine have been made by BARSOUM & GADDUM and CODE. They tested the extract on various biological preparations and found a good quantitative agreement with the results arrived at on the other preparations. Some criticism, however, has been passed on the above methods for the determination of histamine, in which it is maintained that the histidine in the blood is partly decarboxylated by the trichloracetic acid and this would result in too high histamine values. (ÅKERBLOM, 1941).

In summing up it may be said that earlier investigations show that it is possible to make a histamine analysis in blood using BARSOUM's & GADDUM's or CODE's modified method. BARSOUM's & GADDUM's original method seems preferable when analysing whole blood with a low histamine content, while CODE's modification will nevertheless prove advantageous in the analysis of plasma owing to its greater simplicity.

When determining synthetic histamine added to plasma I have followed the principle of the method published by CODE. I have, however, made some small changes in the method of extraction on account of there being a smaller quantity of blood at my disposal for tests than CODE had. I have boiled the preparation with hydrochloric acid of 10 % instead of concentrated acid, since it does not seem to affect the final results, which will be seen from the following experiment. A quantity of 0.77 γ histamine per ml was added to plasma and then the amine

was determined. In three samples boiled with hydrochloric acid of 10 % there remained 0.73, 0.75 and 0.75 γ/ml, in three tests boiled with concentrated hydrochloric acid I found 0.72, 0.73 and 0.78 γ/ml.

The method finally adopted was as follows: A quantity of 6 à 8 ml 10 % trichloracetic acid is added to 3 à 4 ml plasma, and then the mixture is allowed to stand for at least $1^1/_2$ hours. It is then filtered and the precipitate washed 4 times with 3 ml 10 % trichloracetic acid, after which it is mixed with 6 à 8 ml 10 % hydrochloric acid. The mixture is boiled for $1^1/_2$ hours and then evaporated to dryness. Most of the hydrochloric acid is removed through distillation in a boiling water-bath with four portions of 10 ml alcohol. The residue is mixed with 3—4 ml water and a sufficient quantity of Tyrode's solution so that the histamine content in the final test amounts to about 0.3 γ/ml. NaOH is added until the reaction becomes neutral to litmus.

The biological determination on the guinea-pig intestine.

The amount of histamine in the blood preparations produced according to the directions given above have been established by determinations on the small intestine of guinea-pig.

The technique for the determination on the guinea-pig intestine has on the whole been in agreement with the method introduced by MAGNUS (1904) and developed by GUGGENHEIM & LÖFFLER (1916). After killing the guinea-pig by a blow on the head and opening of the blood vessels of the neck — the loss of blood proved advantageous to obtain a sensitive intestine preparation — the front wall of the abdomen was cut away and the small intestine cut off just below the stomach, after which ca. 3 dm of the intestine were taken out and placed in lukewarm Tyrode's solution. Shortly after the intestine had been transferred to a saline solution it began to show spontaneous movements and pieces measuring about 2 à 3 cm were then fixed in a bath measuring 3.5 or 20 ml. After that the spontaneous movements stopped as a rule. The arm of the lever, which transferred the movements of the unfixed end of the intestine to a kymograph, enlarged the contractions of the intestine ca. 6 times. The Tyrode's solution was of the following mixture: 0.8 % NaCl, 0.02 % KCl, 0.02 % $CaCl_2$, 0.02 % $MgCl_2 \cdot 6\ H_2O$, 0.1 % $NaHCO_3$, 0.005 % $NaH_2PO_4 \cdot H_2O$ and 0.1 % glucose. A mixture

of 95 % O_2 and 5 % CO_2 was bubbled through the suspension fluid.

According to BARSOUM & GADDUM and CODE the extracts made on the principles of their histamine determination methods contain choline, and this may prove disturbing to the histamine determination when the biological preparations are particularly sensitive to this substance. Thus CODE stated that an extract tested on the blood pressure of cat shows considerably higher values before the cat has been atropinized. When testing the intestine of guinea-pig this source of error is not considered to exist, though at the suggestion of GADDUM, CODE thinks it advisable to atropinize the intestine as well to lessen its spontaneous movements as to shorten the relaxation time of the intestine. Owing to the fact that the sensitivity of the intestine to histamine is considerably reduced on account of the atropine, the determination of the preparations, short of histamine, may nevertheless be rendered difficult. — I have added atropine (ca. 0.2 γ/ml suspension fluid) to those intestine preparations which had disturbing spontaneous movements or had a long relaxation time.

The intestine preparations, which have been treated as indicated above, have in my experiments reacted to a histamine concentration of down to 0.0006 γ/ml in the suspension fluid. The routine histamine analyses, however, have usually been carried out with a percentage of 0.0015 γ—0.006 γ per ml.

What has been of greater importance than the absolute sensitivity of the guinea-pig intestine for the investigations in this paper has been the possibility to establish differences in the concentrations of histamine on the intestine preparation. FELDBERG & SCHILD (1930) pointed out that this is very slight and they quoted ELLINGER (1930) as having emphasized the fact that it is only possible to distinguish with certainty amounts nof histamine varying from each other with ca. 100 %. Other observers, however, have pointed out that under certain conditions it is possible to show very much better results. For instance TARRAS-WAHLBERG (1936) and CODE (1937 a) pointed out that the intestine of the guinea-pig is sufficiently sensitive to be able to register even slight differences in histamine added, and the latter author goes on to illustrate this by a curve showing that the intestine is able to give clear records of differences right down to below 10 %.

The explanation for these divergent results will most likely

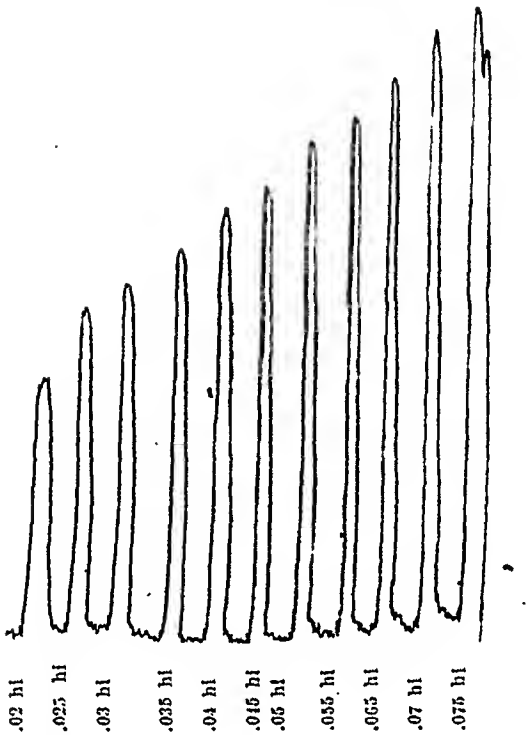


Fig. 2. Contraction of guinea-pig ileum suspended in Tyrode's solution to increasing doses of histamine.

Volume of bath 20 ml. hi = γ histamine dihydroehloride (concentration of amine solution = 0.5 γ/ml)

be due to the fact that the different authors have worked with intestine preparations of extremely varying sensitivity, one and the same piece of intestine from the same animal often reacting quite differently to histamine at different times, in spite of the composition and temperature of the suspension fluid, the tension of the intestine as well as the intervals between the various registrations etc. being kept constant. The reason for the changes in the sensitivity more or less defies explanation. It is possible that during repeated contractions of the intestine preparation metabolic products may form which affect the sensitivity.

Anyone studying this method will soon find how much depends on the way in which the intestine is prepared, how it is kept previous to being hung up, the tension, the frequency of recording and several other details. EICHLER & BARFUSS (1940) thought it advisable to starve the animals for a couple of days previous

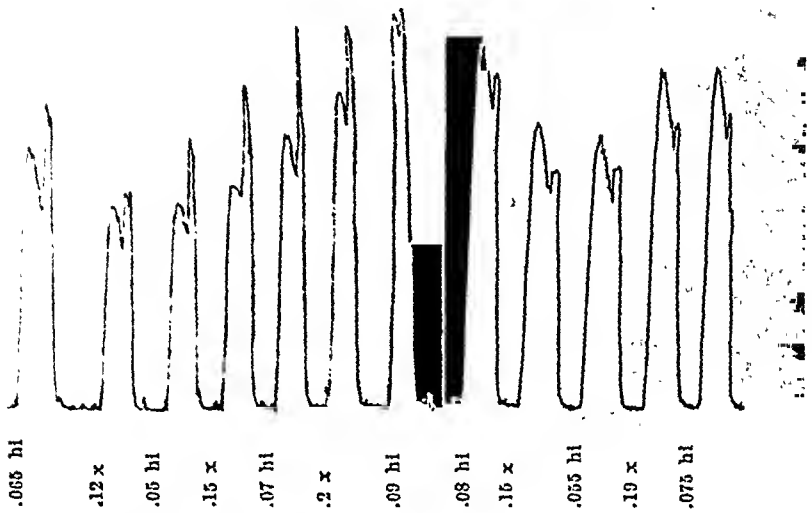


Fig. 3. Quantitative determination on two guinea-pig ileum preparations of histamine in plasma treated according to directions given on page 30. Scale 1 : 1.5.

hi = γ histamine dihydrochloride (concentration of amine solution = 0.5 γ m/l).
x = ml plasma sample.
According to mode of calculation given below, 1 ml x contains 0.42, 0.37 and 0.39 γ when testing on the first and 0.39 and 0.42 γ when testing on the second intestine preparation. Mean: 0.398 γ ± 0.004.

to making use of them for intestine experiments. I have also found that guinea-pigs whose intestines contain only a little usually give better intestine preparations than those whose intestines are loaded. From a material of 29 males and 27 females I could not establish any definite difference between the sexes as regards histamine sensitivity. On the other hand animals weighing between 250 and 400 gr. give somewhat more sensitive intestine preparations than those under 250 gr. — Fig. 2 illustrates that it is possible to get a very high degree of sensitivity in suited preparations.

When determining histamine I added the unknown test to the intestine preparation alternating with a known histamine solution as standard, after which the histamine content of the unknown test has been calculated on the basis of the two histamine records by interpolation according to the following example (fig. 3). In fig. 3 the records no. 3—5 are of the following lengths:

0.05 γ histamine = 34 mm
0.15 x = 37 mm
0.07 γ histamine = 45 mm

From this is calculated:

$$0.15\ x = 0.05\ \gamma + \frac{(37-34)\ (0.07\ \gamma - 0.05\ \gamma)}{45-34} = 0.0555\ \gamma$$

Thus 1 ml $x = 0.37\ \gamma$/ml.

The unknown test is added ca. 6 times to the intestine, the histamine each time being calculated as above, after which the mean of all the calculations is given as the amine content of the test (see fig. 3!).

This method of calculation allows of a sufficiently accurate determination of the histamine. The magnitude of the intestine contractions, however, is not in perfect proportion to the quantity of histamine added, nor does the lever arrangement transfer the movement of the intestine on exactly the same scale in the various parts of the range of the movement. In order to reduce the effect of these factors, however, I have endeavoured to fit in the records of the unknown test between the histamine contractions that differ as little as possible. As a rule the interpolation has taken place between the histamine records, one of which has been up to 20 to 30 % longer than the other. As a rule no interpolation has been carried out when the higher of the flanking records has been caused by a quantity of histamine exceeding the lower by 50 %. As has been shown by EMMELIN, KAHLSON & WICKSELL (1941), who studied the concentration-effect curve for histamine on the ileum of guinea-pig, the most reliable results are obtained when the intestine is brought to contract at between 10 and 60 % of its maximal response. In this rayon the length of the intestine contraction is almost in direct proportion to the quantities of histamine added. I have taken this fact into consideration and have carried out the histamine determinations within the range of this contraction.

As regards the proportionality between the length of the intestine contraction and the record on the kymograph paper, it can be stated that the lever arrangements have been such that no practically significant disproportionality has existed within the range of movement generally made use of.

With this technique the average error varies with different intestine preparations. Here is an example of a series of 18 determinations carried out on two intestine preparations from the same guinea-pig with a Tyrode's solution containing 0.441 γ

Table 1.

Examples showing accuracy with which histamine can be determined in Tyrode's solution containing 0.441 γ amine/ml. Both intestine preparations from same animal. Volume of bath 20 ml.

Intestine preparation no 1		Intestine preparation no 2	
Tyrode's solution added to bath ml	Histamine found γ/ml	Tyrode's solution added to bath ml	Histamine found γ/ml
0.40	0.450	0.40	0.435
0.45	0.411	0.45	0.467
0.40	0.440	0.45	0.429
0.35	0.496	0.40	0.458
0.30	0.373	0.40	0.452
0.25	0.452	0.35	0.466
0.30	0.433	0.30	0.437
—	—	0.25	0.432
—	—	0.25	0.448
—	—	0.30	0.450
—	—	0.33	0.448
Mean: 0.436 ± 0.017 $\sigma = 0.045$; v = 10.5 %		Mean: 0.447 ±0.004 $\sigma = 0.013$; v = 2.9 %	

histamine/ml. The determinations gave the following results: table 1.

Table 1 demonstrates that the histamine determination of one intestine had an average error of 10.5 %, while with the other it was 2.9 %. Both intestine preparations were taken from the same guinea-pig and treated in the same manner, consequently the experiment illustrates the different sensitivity that may be found in different pieces of the same intestine. Moreover, the test with the intestine preparation no. 2 shows how slight the error really can be in some cases with this method of histamine determination.[1]

With 423 determinations of plasma samples prepared according to the method described in this chapter I found an average error of 8.3 % for a single determination, this calculated by means of analysis of variance (SNEDECOR, 1938, BONNIER & TEDIN, 1940). The 423 determinations were carried out on about 30 different intestine preparations. As each plasma sample was estimated on two different intestine preparations, about three determinations on each, the standard error of a sample becomes $8.3 : \sqrt{6} = 3.4$ %.

[1] Most of the routine histamine determinations have been carried out by Mrs M. LINDGREN, to whom I am indebted for her particularly careful technical assistance.

The accuracy of Code's method.

It is interesting to make clear firstly what remains of the amine when using CODE's method, and secondly with what degree of accuracy it can be determined. The method has been systematically tested before by several authors. Thus CODE, EVANS & GREGORY (1938) have found 86—102 % of the added 10—40 γ histamine per liter. ANREP, BARSOUM, TALAAT & WIENINGER (1939) also obtained good yields when adding 66 γ histamine per liter dogs blood. A considerable number of similar tests have also been carried out by EMMELIN, KAHLSON & WICKSELL with a method similar to that of CODE's. They obtained a good yield when adding between 5 and 30 γ histamine base per liter plasma. The latter authors also carried out calculations to see with what degree of accuracy CODE's method can determine native and added synthetic histamine. On the basis of 23 determinations of plasma samples with between 0.03 γ and 0.14 γ histamine/ml they found that the standard deviation was 5.5 % of the average quantity of histamine in plasma.

The experiments mentioned have been carried out on plasma samples with a considerably lower content of histamine than has been used in this paper, and therefore the results obtained cannot simply be considered as proof of equally satisfactory results with higher histamine concentrations. The author has consequently carried out a series of tests with these concentrations, and the results are to be found in table 2 and fig. 4.

Table 2.

Relation between histamine added and found according to method described for histamine determination.

No. of tests	Histamine added γ/ml	Histamine found γ/ml	Difference γ/ml
8	0.016	0.032	+ 0.016
9	0.078	0.103	+ 0.025
5	0.150	0.166	+ 0.016
3	0.290	0.325	+ 0.035
5	0.348	0.340	— 0.008
3	0.491	0.512	+ 0.021
68	0.596	0.573	— 0.023
3	0.714	0.735	+ 0.021
3	0.938	0.954	+ 0.016
3	1.17	1.14	— 0.03
20	1.45	1.42	— 0.03

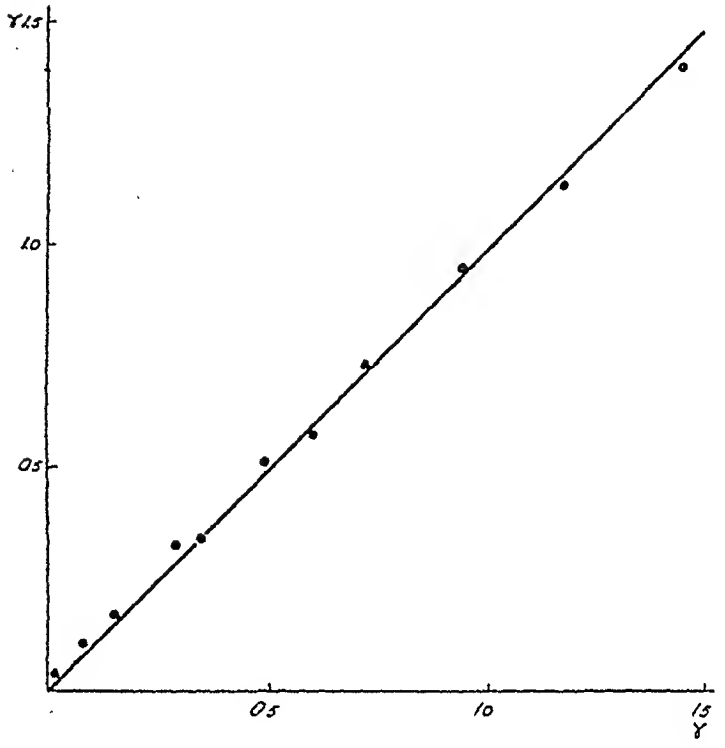


Fig. 4. Relation between histamine added and found according to method described for histamine determination.

Abscissa: histamine added: γ/ml plasma.
Ordinate: histamine found: γ/ml plasma.

A histamine equivalent of 0.014 γ/ml has been found in plasma without any addition of histamine. By adding small quantities I have recovered more than was added, and the difference is made up of what can be presumed to correspond to the amine equivalent in the plasma. If larger quantities are added, however, all is not recovered as a rule, the quantity being a few per cent less than that added. The explanation for this is either to be found in the fact that there has been a real loss of histamine during the chemical treatment or that in spite of this treatment the final preparations contain some substance which decreases the sensitivity of the intestine preparation. The latter hypothesis was tested inter alia by means of the following experiments. Four plasma samples of 4 ml which had been incubated for 22 hours at 37° C so that any possibly existing histamine had been inactivated, were treated according to CODE's modified method. When the preparations had been neutralised 2.43 γ histamine was

added to each. The values of 2.29 γ, 2.57 γ, 2.38 γ and 2.44 γ were obtained at the following determination on guinea-pig intestine when 5 to 6 determinations were carried out on each preparation.

The mean of all the samples was 2.42 $\gamma \pm 0.058$. The experiment shows that the amine really fully corresponds to the histamine indicated in the CODE preparation, and that no inhibitive substance exists. Thus, the decrease in the recovery of the tests, which has been noticed in some cases must be attributed to a loss of histamine during the preparation.

As to the accuracy of the method, the author found in 68 different preparations a coefficient of variation of 6.3 %. The error of the chemical pre-treatment can be calculated from the following. As according to page 35 the intestine determination method has an error of 3.4 %, the standard error of the pre-treatment (σ_{pr}) becomes

$$\sigma_{pr} = \sqrt{6.3^2 - 3.4^2} = 5.3 \ \%.$$

The inactivation of histamine in plasma.

The review of earlier investigations on the histaminolytic power of blood showed that by adding small quantities of histamine to blood and observing the inactivation of the amine on a biological preparation it was possible to obtain an approximately quantitative expression of this power. None of earlier methods published, however, seem suitable for an accurate quantitative judgment of the effect, and consequently a closer investigation has been devoted to kinetics of the inactivation in the blood. I have in this chapter to a large extent made use of blood from pregnant women.

In many cases I have found that histamine in plasma is inactivated with a gradually decreasing rapidity. This is illustrated in fig. 5, in which the inactivation curves for some plasma tests have been inserted. The dots (●) and circles (○) refer to the abscissa stated in minutes, the crosses (×) to the abscissa expressed in hours. The plasma samples come from pregnant women, the dots and circles signifying two subjects during the latter and the crosses a woman during the earlier part of pregnancy.

When it is a question of giving an expression in figures for the rate of inactivation in the reaction it is possible to proceed in two different ways. The first one is to break off the process for all the

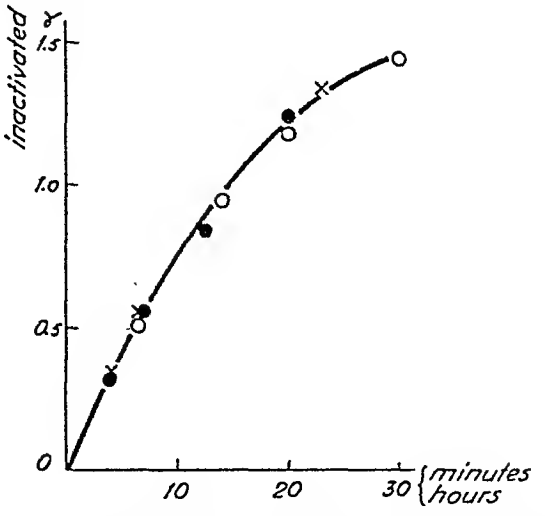


Fig. 5. Inactivation of histamine in plasma from 3 pregnant women. Initial amine concentration 1.5 γ/ml. For explanations, see text.

tests at a definite point of time and examine the remaining quantity of amines, the second is to examine the tests when it is assumed that the reaction has arrived at a certain definite phase.

The former method seems to have been chosen by all earlier scientists, who have made use of the biological method with constant initial histamine percentage, and it seems to give very good results as a rule, if the reaction is broken off at a time when the rate of inactivation is high, i. e. during the first two thirds. On the other hand if the reaction is not broken off until only a small quantity or no histamine at all remains, then only an approximate conception of the histaminolytic power can be obtained. If the reaction has, however, had time to run a very small part of its course when the reaction is broken off, there will be another drawback. The calculation of the quantity of the histamine inactivated is based on the determination of the remaining amine, consequently, if only a tenth part of the histamine has been destroyed when the reaction is broken off and the determination of the remaining amine has been made with an accuracy of, let us say 5 %, then the quantity inactivated cannot be given more accurately than at about 45 %. Thus in the case of plasma tests with very strong and very weak histaminolytic power this method of investigation does not give accurate values.

If the inactivation process is broken off at some definite phase of the reaction in each particular case, it is possible to get a more accurate knowledge of the histaminolytic power. Since the hista-

mine determination is made with the greatest absolute accuracy the smaller the quantity of remaining histamine is, the ideal point of time to break off the reaction theoretically speaking would be just before all the histamine has been destroyed. For practical reasons, however, it is impossible to carry out such an investigation on any great scale, for it presupposes very accurate knowledge of the histaminolytic power in the plasma before the experiment is carried out, and this can only be done if a test has been taken previous to the examination. The author has not carried out such tests as a rule, but has usually broken off the inactivation process earlier instead, that is to say when he has presumed that half of the histamine added has been destroyed. But even this method presupposes a certain previous knowledge of the course of the inactivation in the plasma. Since it is possible — as will be seen later — to calculate approximately the histaminolytic power in the plasma from, amongst others, blood tests that may have previously been taken from the same person, the method which I have suggested will also be practicable when making investigations on a large scale. In order to increase the possibility of catching the reaction just when half of the histamine added is destroyed, I generally take two tests just before and just after what I presume to be this point.

In model tests I have also gone into the question as to whether and to what extent a quantitative calculation can be obtained of an enzyme containing material added to plasma by following the histamine inactivation biologically. Placental extracts with high activity were used as an enzyme source in these experiments. Varying quantities of placental extract have therefore been added to the plasma, after which the disappearance of the histamine has been observed. In order to get a measure for the rate of the inactivation I have determined by means of interpolation the time at which half of the quantity of histamine added can be considered to have been inactivated. Table 3 shows the results of a couple of such tests. In experiment no. 1 plasma was used from a man, in no. 2 from two women: in the tests with 0.29—0.89 mg extract/ml plasma from one woman, and in the test with 17.5 mg extract plasma from another woman. The same placental extract was used for all tests in experiment 2, but another one in experiment 1. Every determination of the time at which half of the quantity of histamine is destroyed is based on an investigation of 2—4 tests taken at different times during the reaction.

Table 3.

The correlation between the amount of placental extract added to plasma and time taken for inactivating half quantity of histamine added. Initial histamine concentration 0.6 γ/ml. For details, see text.

Exp. 1			Exp. 2		
Placental extract added, mg/ml	Time taken to destroy half of histamine added, hours	mg. extract x hours	Placental extract added, mg/ml	Time taken to destroy half of histamine added, hours	mg extract x hours
0.9	4.1	3.7	0.29	13.7	4.0
25.0	0.18	4.5	0.55	6.6	3.6
—	—	—	0.89	3.7	3.3
—	—	—	17.5	0.20	3.5

The tests do show that when adding varying quantities of placental extract to the plasma, there is a histamine inactivating rate during the first half of the process, which roughly speaking is in proportion to the quantity of extract added. If there were any direct proportion between the quantity of added placental extract and the rate of the inactivation of histamine, the product of the extract quantity and the time at which half of the amine is destroyed would be constant when using the same extract. The values found in the table of this product are of the same magnitude, if consideration be taken to the accuracy with which the tests were carried out. Thus the establishment of the rate of inactivation in a blood sample seems under certain conditions to be able to give an approximate expression of the histaminolytic power existing in the blood.

It was also found that the inactivation of histamine by placental extract in plasma from different subjects proceeded with the same rate, which is illustrated in fig. 6. The reason why the values in fig. 6 of inactivated histamine show greater variation at the beginning than at the end of the test is no doubt due to the fact that the determination of the remaining quantity of histamine proceeds with greater accuracy if there is less histamine left in the test. This experiment indicates that plasma tests inactivating histamine with the same rate contain an equally great histaminolytic power. Thus any possible existing enzyme activators and inhibitors which may affect the course of the reaction seem to exist in the same quantities in different samples of plasma. This conclusion is also supported by some experiments

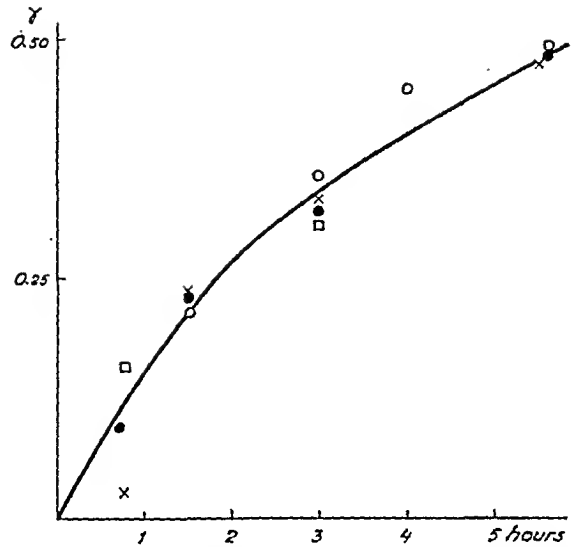


Fig. 6. Inactivation of histamine by placental extract in plasma from four different subjects. 2.9 mg placental extract is added to one ml plasma. Initial histamine concentration 0.6 γ/ml. ●, ○ and □ denotes plasma samples from three women, × from a man.

Abscissa: incubation time.
Ordinate: inactivated histamine per ml.

in which I have mixed plasma samples of different histaminolytic power and then investigated this power in the mixtures (table 4). The table shows that the histamine destroying effects of the mixtures are in good agreement with those calculated.

Table 4.

Estimation of histaminolytic power in mixtures of different plasma samples.

Exp. no.	Plasma sample 1.		Plasma sample 2.		Plasma sample 3.		Calculated histaminolytic power of mixture γ/ml/h	Histaminolytic power found, γ/ml/h	Difference %
	ml	γ/ml/h	ml	γ/ml/h	ml	γ/ml/h			
1	5.0	3.2	13.0	4.2	11.0	1.3	2.9	3.1	+7
2	12.5	0.12	10.5	0.31	—	—	0.21	0.20	−5
3	6.0	2.7	6.0	1.9	—	—	2.3	2.4	+4

In order to establish the rate of the histamine inactivation in plasma the first thing to do was to try to establish a suitable initial histamine concentration in the reaction mixture. Experiments with the addition of placental extract to plasma had indicated that with a histamine concentration of 0.6 γ/ml, it was possible

to show small quantities of the extract by investigating the inactivation. Earlier investigators in this field [WERLE & EFFKEMANN (1940 a), ANREP & al. (1941) amongst others] have also found an initial histamine concentration of about 0.6—1 γ histamine base to be suitable. Therefore I chose this concentration at first, but on finding that certain blood tests showed such a high histaminolytic power that it took only a few minutes to inactivate half of the amine, I increased the concentration to 1.5 γ/ml.

There was another reason that made me change and make use of 1.5 γ/ml. As will be seen in fig. 4 (p. 37), when determining the histamine in concentrations under 0.3 γ/ml, higher values than those added are as a rule obtained. Therefore in order to form an idea of the difference in the histamine percentage, an amine determination must in consequence be carried out as well at the beginning as at the end of the test to obtain by means of subtraction the most correct value possible of the quantity of histamine inactivated. This source of error is much less with higher histamine concentrations. The quantity of histamine added does not differ from that obtained with the intestine determination by more than a few per cent, consequently it is unnecessary to test the histamine content at the beginning, which means much less work. I have carried out tests with slight histaminolytic power either with the higher or lower initial histamine concentration or with both, all according to which has been most suitable with consideration to the time at which the incubation had to be broken off. In 10 tests when the determination of the histaminolytic power was carried out at the same time with both the initial concentrations, I found on an average 10—15 % less values of the histaminolytic power with the 0.6 γ/ml than with the 1.5 γ/ml concentration. I have not corrected the values with reference to this relation, since the source of error thus caused in relation to the absolute variations in the histaminolytic power of the subjects concerned — pregnant women in the 7th to 10th weeks — is negligible. As we shall see later the histaminolytic power increases by about 2000 % during this period.

Method for the determination of the histaminolytic power.

The examination has usually been carried out in the following manner: 13 to 17 ml heparinized plasma is incubated in a little

round retort for about 5 minutes in a water bath at 37° C. After that 0.65 to 0.85 ml histamine solution is added containing 30.4 γ histamine base pr ml in phosphate buffer solution at pH 7.2. As soon as the histamine has been added, the retort is shaken thoroughly so that the plasma and histamine get properly mixed and at the same time a stop-watch is released. When the reaction is to be broken off a syringe is put into the reaction mixture having a glass capillary tube of about 5 cm at the end. By means of an adjustable brake-control fitted on to the piston and with a quick movement of the hand the tube is filled with an exact quantity of the fluid, usually 3 or 4 ml, after which it is spurted into double the volume of 10 % trichloracetic acid so that the histamine inactivation is broken off. It takes about 5 seconds from the moment when the fluid, is drawn up into the tube till it is precipitated in the trichloracetic acid. The actual time of incubation is generally given to within 6 seconds, with longer tests to within 1 minute. About four tests are taken at every experiment.

Plasma tests, which under the conditions now mentioned inactivated less than half of the amine added in 5 or 6 hours, have only been supplied with 0.26 to 0.34 ml amine solution to 13—17 ml plasma instead of 0.65 to 0.85 ml histamine solution.

When the precipitated plasma tests have stood for $1^1/_2$ hours they are treated as is earlier described and the quantity of histamine is determined on the small intestine of guinea-pig.

If the values obtained for the remaining quantity of histamine are subtracted from the quantity added and inserted in a coordinate system, the inactivation curves are obtained, some typical examples of which are given in fig. 7. In order to get representative expression from the values obtained of the inactivation rate in the different tests, I have proceeded in the following manner. All the values showing an inactivation of between 33 and 67 % of the amine added have been put together and the mean has been calculated. Then the mean for the times at which the same tests were taken has been calculated, and the averages thus obtained for inactivated histamine and inactivation time have been used to form the basis for judging the histaminolytic power. This has been expressed as *the quantity of histamine base inactivated by 1 ml plasma in the course of one hour* ($\gamma/ml/h$).

Earlier units given for the histaminase determination may be mentioned here. A unit originally proposed by BEST & MCHENRY was established from the activity essential for the destruction of

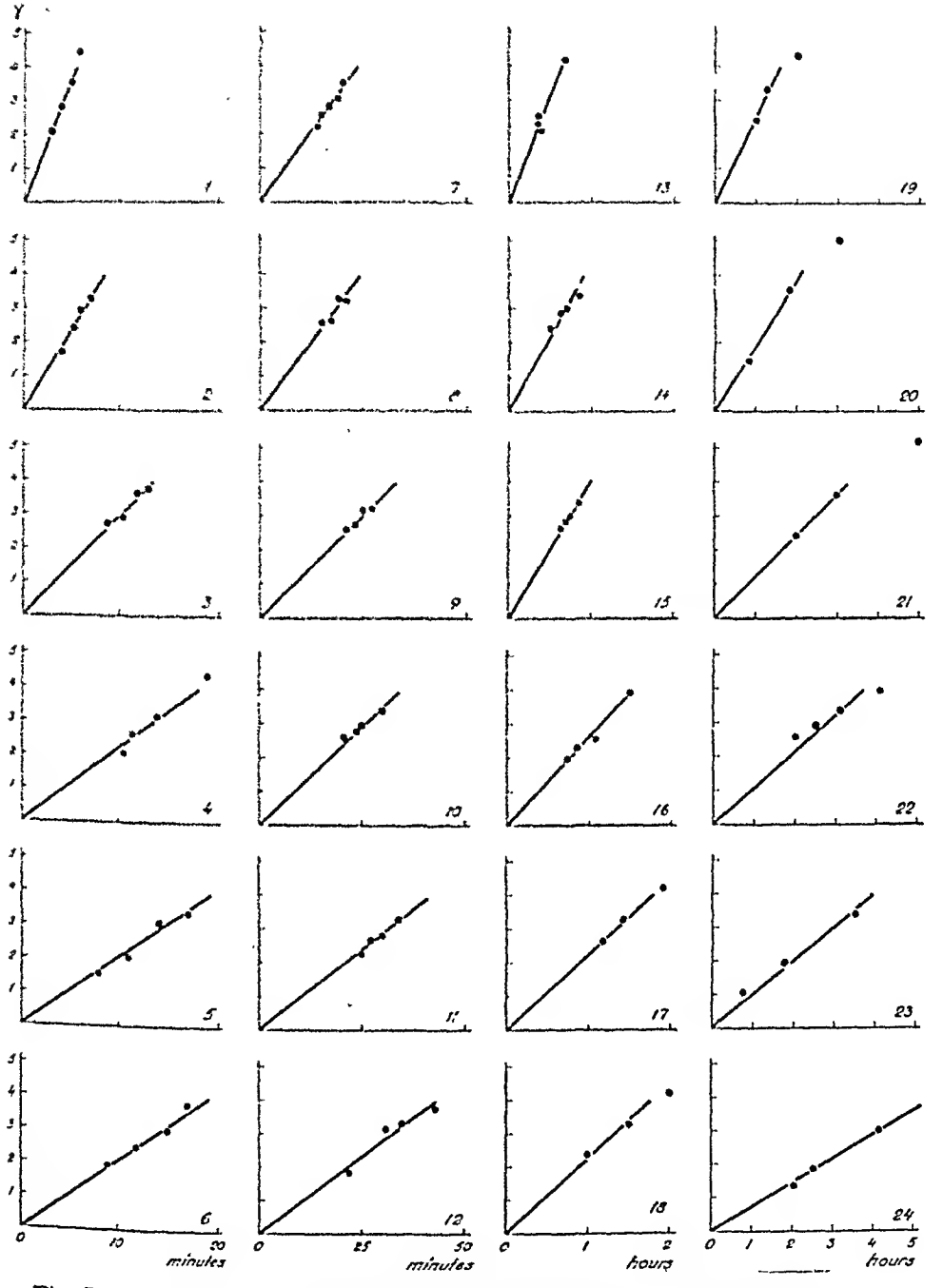


Fig. 7. Inactivation of histamine in 24 different plasma samples. Initial amine concentration 1.5 γ/ml.

Abscissa: incubation time (minutes and hours in respective columns of curves). Ordinate: inactivated histamine, expressed in γ per 4 ml of incubation mixture. The diagonal lines are drawn through origo and the mean of the values lying between 2 and 4 γ.

1 mg histamine during 24 hours' incubation at 37° C in a phosphate buffer solution at pH 7.

The diamine oxidase unit proposed by ZELLER, BIRKHÄUSER, MISLIN & WENK (1939) indicates the activity in an enzyme solution, which saturated with cadaverine, brings about an initial metabolism of 10^{-6} mol per hour. This unit with various modifications has been used by several authors. Another unit has recently been adopted by STEPHENSON (1943). He described a unit as the quantity of enzyme necessary for catalyzing the consumption of 1 c.mm. oxygen in 10 minutes at pH 7.0 and at 37.5° C in a solution containing 0.1 M ammonium ion and 1 m/M histamine. The histaminase unit of BEST & MCHENRY (1930) corresponds to a destruction of 40 γ histamine per hour, a diamine oxidase unit of ca. 110 γ and a unit of STEPHENSON's of about 60 γ. ZELLER's unit has been calculated with histamine as the substrate and no consideration has been taken to the influences that the different ions and temperatures postulated at the various standardizations cause. Should cadaverine be used instead of histamine at the enzyme determination according to ZELLER, the ratio of enzyme obtained must on account of the greater reaction rate of the cadaverine be divided by 1.3 in order to be able to be compared with the units based on histamine.

The conditions under which I examined the blood deviate in several respects from those stipulated for the units above mentioned. It therefore seemed practicable simply to express the histaminolytic power of plasma in the quantity of histamine destroyed by one ml plasma in an hour according to the conditions indicated above.

The schematic process of calculation used by the writer presupposes that the inactivation of the remaining second third of the histamine added takes place with constant or almost constant rate. As has just been mentioned, however, the inactivation generally takes place with a gradually decreasing rate. During the time while the second third of the histamine is being destroyed, however, the inactivation (see curves in fig. 7) takes place according to an almost rectilinear course. If the inactivation is calculated on the basis of the values corresponding to 33 % of the inactivation, a somewhat higher expression is usually obtained than if calculated on the values corresponding to the 67 % inactivation. In order to avoid this inconvenience the author has endeavoured as far as possible to suit the times of incubation so that

two values correspond to between 33 and 50 % of the inactivation, and the other two to between 50 and 67 %. By doing so the mean for the four tests is intended to come very close to 50 %, thus considerably reducing the above mentioned inconvenience.

As the histaminolytic power in the plasma samples has not been known in detail previous to the carrying out of the experiments, however, it has not always been possible to suit the times of incubation so that fitting values of inactivation have been obtained. A material of 164 determinations of the histaminolytic power selected at random showed, however, that the mean inactivation had been calculated on a value corresponding to 49.7 % of the inactivation of the amine and that 2/3 of all the values were obtained between 44 and 56 %. As the work proceeded and I gained greater experience as to the histaminolytic power of the blood, however, I was able to prognosticate with greater certainty the rate of inactivation in the various blood tests. By this means it was possible to choose the times of incubation so as to get nearer the time when half of the histamine was destroyed, and thus still further reduce the importance of the sources of error referred to.

The author has tried to make out of how great importance this source of error is for the accuracy of the inactivation equivalents. By means of graphic calculation of a number of inactivation curves, however, I have found that in 2 cases out of 3 the expression of the histaminolytic power has an error less than 3.5 to 4 %. In 31 cases when 0.6 γ/ml was used as the initial histamine concentration, by the same calculation I found that the error indicated in 2 cases out of 3 was at the most 13 %. Taking into consideration that the variations in the histaminolytic power of blood — as expressed by means of the method used in this work — can amount to hundreds of times during pregnancy, the error just referred to is negligible.

Another source of error when calculating the histaminolytic power lies in the fact that the amine shown in the plasma tests does not correspond to what is found in the test immediately before it is precipitated with trichloracetic acid. As has been previously pointed out it has been possible to show by recovery tests that the histamine equivalents obtained usually very closely agree with the quantity of histamine existing in the test. Table 2, however, shows that with a histamine percentage of 0.6 γ/ml a small percental loss must be reckoned with. It has not been definitely established whether this is also the case with

higher histamine percentages, but the values in the table indicate that it may be so. The high values after adding 0.7 and 0.9 γ/ml indicate, however, that the loss is of no great importance. If the 77 tests in table 2 in which 0.45 to 0.98 γ/ml is added — which are the limits between which the histamine remains when determining the histaminolytic power — are summarized, an average loss is obtained of 0.018 γ/ml. If consideration is also taken to plasma's own histamine, which on an average may be calculated at 0.02 γ/ml, it seems possible to be able to reckon with a loss of about 0.038 γ/ml at the histamine determination, corresponding to an error of at most 5 %. The investigations carried out, however, do not seem to be so complete that they allow of a consistent addition of 0.038 γ histamine per ml test to obtain the correct value.

If full consideration should be taken to the above mentioned facts when determining the histaminolytic power, it would be necessary to establish the probable histamine recovery in each particular case by means of tests with the plasma in question during the experiment. The author has made it a rule to carry out such control tests when it has been a question of establishing slight differences in the histamine contents of the plasma tests, but as will be understood from the above, he has not carried them out with the ordinary test of the histaminolytic power. In one case, however, simultaneously with the inactivation, I have examined the histamine recovery with the amine concentrations that were left after the different times of incubation. In three series of experiments each consisting of five plasma tests there was added about as much histamine as was left in the tests incubated. If the tests in the series incubated were corrected bearing in mind the mixture experiments, and the histaminolytic power were calculated in the ordinary manner, it was found that the valuc corrected in this case differed from the non-corrected by 2.7 %.

Considering the slight magnitude of the error as compared with the great changes in the histaminolytic power during the various phases of pregnancy, the author has not studied the effect of this source of error on the histaminolytic power more closely.

The accuracy with which it is possible to determine the histaminolytic power of plasma according to the method now described depends on 1: The accuracy of the histamine determination.

Table 5.

Repeated determinations of the histaminolytic power of plasma. "No. of plasma samples" indicates the number on which the determination is based in each particular case. Each plasma sample is based on about 6 determinations on the intestine.

Exp.	Test no. 1		Test no. 2		Test no. 3		Mean	Coefficient of variation %
	No. of plasma samples	γ/ml/h	No. of plasma samples	γ/ml h	No. of plasma samples	γ ml/h		
1	4	0.382	4	0.376	4	0.395	0.384	2
2	3	4 11	3	4.26	2	3.92	4.10	4

2: How nearly it is possible to suit the time of incubation to the moment when half of the histamine has been inactivated, 3: How many tests have been taken at that moment, and 4: Measuring and other errors.

Ad 1 and 3. Acccording to p. 38 the histamine content in the plasma tests is determined with an average error of 6.3 %. As about 3 plasma samples are taken as a rule to form an average determination of the histaminolytic power, the variation of the histamine determination causes an average error of $6.4 : \sqrt{3} = 3.7$ %.

Ad 2. The source of error here discussed causes an average error of 3.5 to 4 % (p. 47).

Ad. 4. Other causes for the variation in values have not been calculated in detail, but are presumed to be approximately 5 %, and therefore the average error for the determination of the histaminolytic power > 0.12 γ/ml/h can be estimated as being $\sqrt{3.7^2 + 3.75^2 + 5^2} = 7.3$ %.

According to a corresponding calculation for the values between 0.014 and 0.12 γ/ml/h an average error is obtained of approximately 16 %.

As an example of the possibility in particular cases to determine the histaminolytic power with an error of only 2—4 %, I should like to give the following, where two different plasma samples were examined three times each (table 5).[1]

Keeping blood tests etc. As soon as the blood tests had been taken from the cubital vein in a glass flask with a few drops of

[1] I am greatly indebted to Dr L. Goldberg, Pharmacological Department, Caroline Institute, for his valuable advice concerning the statistical treatment of the material.

2 % heparine solution they were immediately centrifugated and kept at + 4° C. The histaminolytic power does not change when keeping plasma for some days under these conditions. The histaminolytie power of serum is only slightly lower than that of plasma, which means that even if the tests had coagulated in spite of the addition of the heparine, they permit of an approximate determination of the histaminolytic power. The addition of heparine to serum does not change its histaminolytic power.

The histaminolytic power of whole blood is about half that of plasma. Slight hemomolysis in plasma does not bring about any change in the histaminolytie power.

Some characteristics of the histaminolytic principle.

According to ZELLER, BIRKHÄUSER, MISLIN & WENK (1939) as well as WERLE & EFFKEMANN (1940 a) the histaminolytic power of the blood is to be attributed to the effect of histaminase or diamine oxidase, the former authors finding that serum mixed with cadaverine caused oxygen uptake, the latter that certain substances inhibiting the histaminase brought about an inhibition of the histaminolytic effect of the blood. In order further to illustrate the relation between this power of the blood and the histaminase I have carried out investigations on some of the physieal properties.

The histaminolytie power of plasma completely disappears when heated up to 73 or 74° C for 3½ minutes. If not heated to more than 69° or 70° C only about half of the activity is destroyed in the same time. If on the other hand plasma is kept at 37 °C for 24 hours its power to inactivate histamine is not reduced.

The observations made seem to be in good agreement with earlier statements concerning histaminase (BEST & MCHENRY, SWEDIN 1944), according to which heating up to 60° to 80° C causes inactivation of the enzyme.

According to the investigations of BEST & MCHENRY the activity of their kidney powder histaminase had its optimum temperature at 37° C. I have found that histamine in plasma is inactivated more quickly at a higher temperature than 37° C, for instanee, by incubating a histamine—plasma mixture up to 57.8° to 58° C for 24 minutes I could observe an inactivation of the histamine which was twice or three times

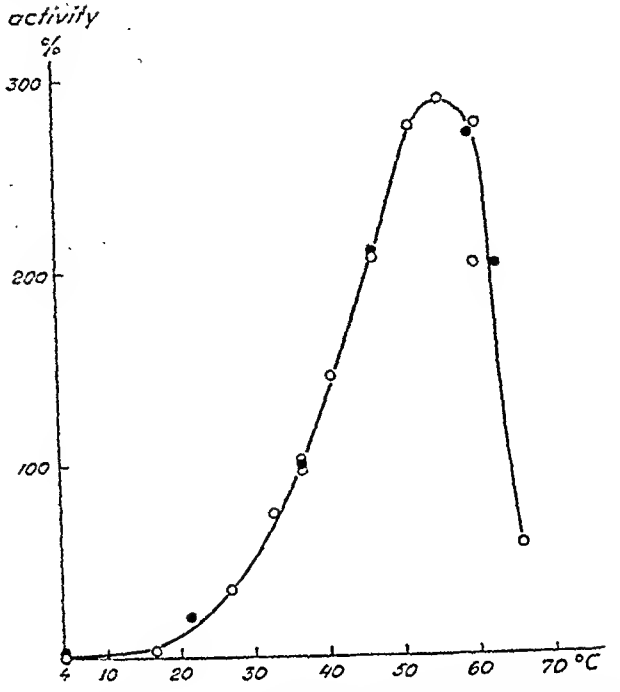


Fig. 8. Histaminolytic power of plasma from pregnant women (●) and placental extract (o) at different temperatures.

Abscissa: incubation temperature.
Ordinate: histaminolytic activity expressed in per cent of that at 37° C.

more rapid than at 37.0° to 37.5° C. Higher temperatures than 58° C reduced the rate of the inactivation. When the temperature went under 37° C the rate was lower than at 37° C. Fig. 8 shows a graphic registration of the inactivation rate at some different temperatures expressed in % of the inactivation at 37° C. For cases with a temperature of >37° C the incubation has not been followed for more than 36 minutes, at 37° C for 1 hour 2 minutes and for <37° C up to 10 hours.

It is a well known fact that many conditions, first and foremost the degree of purity, affect the optimum temperature of enzymes. Thus the fact that a different optimal temperature is found in plasma from what BEST & MCHENRY found in their histaminase extract need not necessarily be inconsistent with the fact that the histamine inactivating principle of plasma is made up of histaminase.

The optimal hydrogen ion concentration was at pH 6.8—8 for BEST & MCHENRY's histaminase powder. In SWEDIN's purified extracts the enzyme was rendered partially inactive at

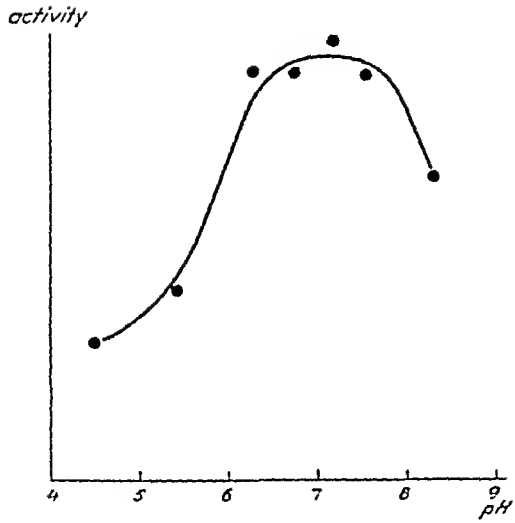


Fig. 9. Histaminolytic power of plasma from pregnant women at different pH.

pH 5.6. In an experiment with plasma samples diluted with phosphate buffer solution at different pH, I found approximately the same histamine inactivation between pH, 6.3 and 7.5. The reaction rate is reduced (fig. 9) with lower or higher pH values than these, and this seems to be in agreement with what earlier observers have found with regard to histaminase.

The destruction of histamine and diamines by means of different histaminase preparations proceeds in a somewhat different manner depending on how the preparation is produced. After a lag phase the inactivation begins with a rate which is at first high and afterwards decreases. BEST & MCHENRY and MCHENRY & GAVIN (1932) found under certain conditions a lag phase lasting for 5 hours, while ZELLER (1938 a, b) and KIESE (1940) observed a latency of only a few minutes. ZELLER, SCHÄR & STAEHLIN (1939) could not show any lag phase with a phosphate buffer solution of dry acetone powder, the oxygen consumption beginning immediately after the substrate had been added. Nor did SWEDIN observe any lag phase with his purified preparation. WERLE and EFFKEMANN (1940 a) observed no lag phase either when investigating the histamine inactivating effect of blood.

My investigations indicate (fig. 5 and 7) that the histamine inactivation of plasma begins practically immediately after the plasma has been mixed with the amine.

Varying information exists, too, as regards the further course of the reaction. MCHENRY & GAVIN (1932) and ZELLER et al.

considered that they had established the fact that the destruction takes place in the same manner as a monomolecular reaction, while KIESE often found a continually increasing rate during the reaction. According to ZELLER (1938 a), however, the reaction takes place with a constant rapidity until two oxygen atoms have been consumed per molecule substrate. With his preparation purified by means of electrophoresis SWEDIN found a constant rate of destruction. According to WERLE & EFFKEMANN the rate is constant throughout the course of the inactivation in the blood. As has previously been pointed out, I have found that the inactivation in plasma takes place with a gradually decreasing rate.

The investigations which have been carried out on the histamine inactivating principle support earlier observations as regards the existence of a histaminolytic enzyme in blood. From the earlier statement, however, it seems clear that at least two separate enzymes or systems of enzymes probably influence the inactivation of the histamine (SWEDIN 1944). It is evident from the experiments just alluded to by ZELLER et al. that the component oxidatively deaminating the side chain is to be found in serum, though no investigations have been carried out to illustrate the existence of the factor in plasma which breaks the ring. Thus, further investigations are necessary to decide the question as to the nature of the histaminolytic principle of plasma.

CHAPTER IV.

The histaminolytic power of blood in animals.

Guinea-pigs.

Investigations as to the histaminolytic power of guinea-pigs blood have already been carried out by BUSSON & KIRSCHBAUM (1912) and YEN & CHANG (1933) with negative results. MARCOU (1938) found that 4.5 γ out of 30 γ histamine phosphate were destroyed if incubated with 5 or 10 ml guinea-pigs blood for half an hour at 37° C.

Based in principle on the same method as has been described on page 43, the author has investigated the histaminolytic power of guinea-pig blood. It had to be modified in some respects, however, owing to the shortage of blood obtainable for the purpose. In those cases where repeated blood tests were taken on the same animal, blood was used instead of plasma. A quantity of 7.5 ml phosphate buffer solution as well as 1.5 ml of a histamine solution containing 3 γ histamine per ml was added to 1.5 ml blood — taken by means of heart puncture. Tests of 4 ml were precipitated with trichloracetic acid at the beginning of the incubation as well as 4 or 5 hours later, and they were then treated according to the modification of CODE's method, which has already been described (page 30), after which the histamine was estimated on the intestine of guinea-pig. In a few cases a test was also taken after 2 hours. The histaminolytic power was expressed in γ of inactivated histamine base per ml blood and hour on the basis of the changes observed in the histamine of the samples.

A report will now be given of the experience gained from a number of animals. When examining 8 male guinea-pigs, from which 18 tests were taken, I found a histaminolytic power cor-

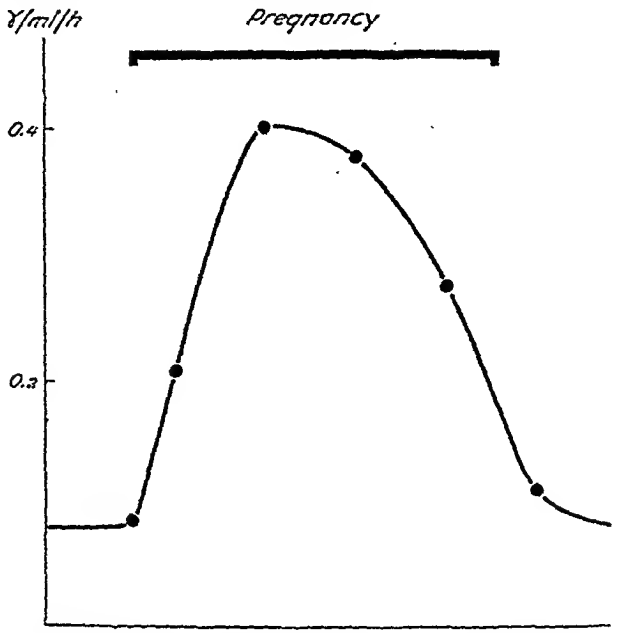


Fig. 10. Histaminolytic power in guinea-pig blood during pregnancy. For explanations, see text!

responding to 0.24 γ/ml/h± 0.03 (max. 0.5, min. 0.0 γ/ml/h). In 20 tests from 7 virginal animals a histaminolytic power was established of 0.08 γ/ml/h ± 0.03 (max. 0.2, min. 0.0 γ/ml/h), and in 24 tests from the same animals taken after they had become pregnant the results were 0.31 γ/ml/h ± 0.02 (max. 0.8, min. 0.0 γ/ml/h). Tests of the histaminolytic power during various phases of the pregnancy were carried out on these animals. The results of this investigation is to be seen in fig. 10, where the blood tests taken are averaged for every half month during the pregnancy. The figures are based on 5 tests during the first half month, 7 during the second, 4 during the third and 8 during the last half month of the pregnancy. In 9 tests taken within 2 weeks of parturition a mean histaminolytic power of 0.11 γ/ml/h was established.

The investigations carried out show that under certain conditions there is a histamine destroying effect in guinea-pigs blood, and all males examined have shown this effect. I have found a striking difference in the histaminolytic power in pregnant and non-pregnant animals. The virginal animals examined showed only a slight histaminolytic power, which increased during pregnancy and reached a maximum after a month or so, whereupon the effect diminished somewhat towards the end of the

time. The variations observed during the course of the pregnancy, however, are based on so few determinations that they can be only considered to give a rough estimation of the actual facts. After parturition the values seem to drop again in the direction of those observed in virginal animals.

The difference shown in the histaminolytic power in males and females is statistically significant, though the investigations made do not allow of any more detailed particulars as to the degree. ZELLER, STERN & WENK (1939) have shown how the diamine oxidase of the kidney increases during the growth of the organism, and the difference might therefore only be due to the difference in the degree of development in males and females. All the animals examined, however, were from the same stock and were about the same age and weight, and therefore the difference observed must be attributed to the sex.

The contradictory results obtained in the course of earlier investigations of the histaminolytic power of guinea-pigs blood may possibly be attributed to the fact that different authors have not taken into consideration the importance of the sex — and in the case of females — pregnancy.

Rats.

ROSE, KARADY & BROWNE (1940), who studied the effect of the administration of histamine on the histaminase content in the various organs of rat, also examined the blood. A quantity of 800 γ histamine was added to 10 ml oxalated blood, after which the mixture was incubated at 36° C for 16 hours and then treated according to a modification of BARSOUM's & GADDUM's (1935) histamine determination method adopted by CODE (1937). No destruction of the histamine could be proved by this method.

The author examined the histaminolytic power in heparinised plasma from about 20 rats. The blood used was taken after the decapitation of the animals, and the examination was carried out as follows. A quantity of 3 ml plasma was incubated at 37° C with 15 ml of a solution containing 0.486 γ histamine per ml in phosphate buffer solution. The histamine was determined in 3 samples of 5 ml taken when the incubation was begun, after about 1 hour and after about 5 hours. The histaminolytic power was expressed as the quantity of histamine base inactivated by 1 ml plasma per hour.

On examining the plasma from 2 males and 5 virginal rats no definite inactivation could be proved under the conditions prevailing, but from 14 pregnant rats I found an average histaminolytic power of 0,15 γ/ml/h ± 0.02 (max. 0.29 γ/m/h, min. 0.06 γ/ml/h). The individual values for 12 of these rats are given in table 12, page 94.

The examination shows that plasma from pregnant rats inactivates histamine, whereas no inactivation could be proved from non-pregnant animals and males. ROSE's, KARADY's & BROWNE's (1940) negative results may probably be due to their not having examined blood from pregnant animals.

Rabbits.

YEN and CHANG (1933) found that a quantity of between ca. 3 and 30 ml rabbits blood is required to inactivate ca. 0.6 mg histamine phosphate in 24 hours. MARCOU (1938), however, could not establish any histaminolytic power of rabbits blood. WERLE & EFFKEMANN (1942) examined blood from non-pregnant and pregnant rabbits; they found no inactivation of 9 γ histamine incubated with 3 ml blood or serum for 1½ hours.

I have examined the histaminolytic power of plasma from rabbits. Blood was taken by means of a heart puncture with a syringe containing about one drop of 2 % heparine solution. The tests were carried out in the following manner: a quantity of 4.5 ml phosphate buffer solution and 0.45 ml histamine solution containing 30.4 γ/ml was added to 4.5 ml plasma. The mixture was incubated for 5 or 6 hours at 37° C. Samples of 3 ml were examined according to the method described on page 30 immediately after the histamine had been added as well as after about 3 hours and also at the end of the incubation. The histaminolytic power was calculated on the basis of the decrease of the histamine observed in the plasma and expressed as γ inactivated histamine base per ml and hour.

An examination of 6 plasma samples from 5 male rabbits showed an average histaminolytic power of 0.29 γ/ml/h ± 0.02 (max. 0.38 γ/ml/h, min. 0.21 γ/ml/h). In 7 plasma samples from 5 virginal rabbits I found an average histaminolytic power of 0.21 γ/ml/h ± 0.02 (max. 0.27 γ/ml/h, min. 0.07 γ/ml/h) and in 8 plasma samples from 3 pregnant animals — taken at various

stages of pregnancy — I found 0.19 γ/ml/h ± 0.04 (max. 0.31 γ/ml/h, min. 0.00 γ/ml/h).

The investigations showed that plasma from rabbit is able to inactivate histamine. It has not been possible to show any definite difference in this plasma power from male and female rabbits, nor have I been able to observe that it undergoes any change during pregnancy.

The results confirm YEN's & CHANG's investigations. The fact that WERLE & EFFKEMANN and MARCOU have observed no histaminolytic power in rabbit blood may possibly be due to their having incubated their preparations for so short a time ($1^1/_2$ hr and $^1/_2$ hr) that the inactivation was not sufficient to make itself apparent.

Cattle and other animals.

Earlier investigations have been carried out by WERLE & EFFKEMANN (1942) according to their method referred to on page 18. They found in serum from cows a constant histaminolytic effect, which they considered to be equally great both in pregnant and in non-pregnant animals.

Table 6.

The histaminolytic power of plasma from cattle. The blood from the first four animals taken at time of slaughter, others by venesection.

Sex		γ/ml/h
Bull		1.0
»		1.6
Cow	non pregnant	1.3
»	» »	1.3
»	» »	1.1
»	4 months after covering	1.6
»	7 » » »	1.0
»	10 » » »	2.0

With the method adopted for man I have examined the blood from 2 bulls and 6 cows, three of which were pregnant. The results of these investigations are to be found in table 6, from which it will be seen that there is a considerable histaminolytic power in plasma from both bulls and cows. It cannot be definitely stated with these investigations whether there is

any increased enzyme activity during the pregnancy, but it cannot be entirely excluded. This conclusion is in no way contradictory to the results referred to by WERLE & EFFKEMANN. When examining the results of these authors' investigations more closely, we see a similar tendency to a slight increase among the pregnant animals. Further investigations, however, are necessary in order to be able to decide this question.

Occasional blood tests have been taken from other large domestic animals at the slaughter. The tests have then been examined according to the method adopted for humans.

Thus two experiments have been carried out with plasma from a horse (castrated male). In one case the histaminolytic power corresponded to 0.3 γ/ml/h, in the other it was somewhat greater than 0.2 γ/ml/h. The histaminolytic power in a ram and a ewe was 1.1 and 1.0 γ/ml/h respectively. A plasma test from a non-pregnant sow showed a somewhat higher value than 3γ/ml/h.

WERLE & EFFKEMANN (1942) have found an average inactivation in normal and pregnant horses of 0,5 to 1.0 γ of histamine dihydrochloride per 3 ml serum during $1^1/_2$ hours, which is of the same magnitude as my results. Furthermore these authors found that hog serum inactivated 2.0—2.5 γ under the same conditions, which is somewhat less than my value.

In the serum from a non-pregnant cat I could not observe any histaminolytic power, though from a pregnant cat I found a histaminolytic power corresponding to about 0.004 γ/ml/h.

These investigations were intended to form an introduction to the physiology of the histaminolytic power of blood in some animals with special reference to pregnancy. If the results are summed up, it will be seen that the males and non-pregnant females from all the animal species examined show signs of histaminolytic power with the exception of the rat. An increase of this power has been established in rat and guinea-pig during pregnancy.

Although the absolute values of the histaminolytic power are not directly comparable owing to the various methods used when carrying out the investigations, it may nevertheless be said that cattle, sheep and hog show a considerably stronger effect than guinea-pig, rabbit and horse.

CHAPTER V.

The histaminolytic power of plasma in man.

A. Men and non-pregnant women.

The following method was adopted when examining the histaminolytic power in men and non-pregnant women. A quantity of 0.6 γ histamine was added to each ml of plasma, after which the remaining quantity of histamine was determined after incubation for 22 hours at 37° C. For the sake of control 4 plasma samples taken immediately after the histamine had been added were examined as a rule, and after 22 hours another 4 samples were usually examined. All the 8 tests were treated according to the method described on p. 30, and then the histamine determination was carried out on intestine about 6 times per sample. The difference in the histamine content at the beginning and the end of the incubation was then calculated and the histaminolytic power expressed in the manner already stated.

An examination of 11 tests from 7 healthy men showed an average of 0.006 γ/ml/h $\pm$ 0.001, ($\sigma = 0.004$, max. 0.016 γ/ml/h, min. 0.002 γ/ml/h).[1]

An examination of 25 separate blood tests from 13 healthy non-pregnant women showed an average histaminolytic power of 0.005 γ/ml/h $\pm$ 0.0006 ($\sigma = 0.003$, max. 0.015 γ/ml/h, min. 0.002 γ/ml/h).[2]

This histamine cleavage in men and non-pregnant women is of such a low magnitude that it requires a particular analysis. The question that first arises is whether an inactivation of such a

[1] Test subjects, 20—35 years of age, have been medical students and colleagues.

[2] Test subjects, 20 to 40 years of age, have been medical students, assistants at the Pharmacological Laboratory of the Caroline Institute and at the Department of Industrial Hygiene of the State Institute for Public Health as well as personal friends.

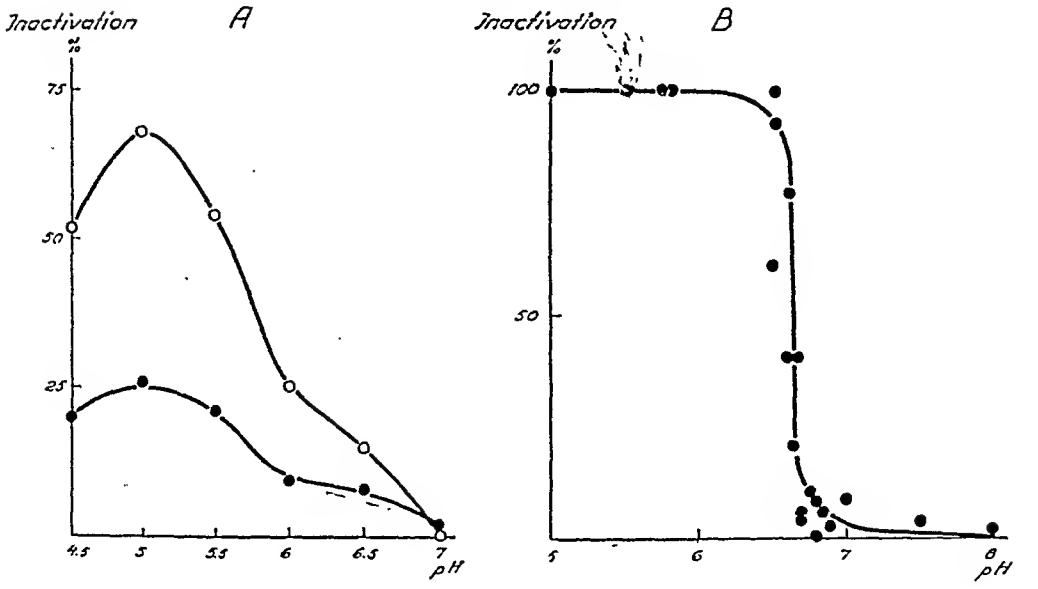


Fig. 11. Bacterial inactivation of histamine in m/15 phosphate buffer solution at different pH.

A: Initial conc. of histamine = 6 γ/ml. ● = incubation for 12 hours. ○ = incubation for 17 hours.

B: Initial conc. of histamine = 0.6 γ/ml. Incubated 40 hours.

degree can be wholly explained by a spontaneous decomposition of histamine at the temperature used. In order to test this the following experiment was carried out.

A sterile histamine solution was added to Tyrode's solution under sterile precautions. Half of the histamine solution was kept at + 4° C, half at 37° C for 22 hours. After this time the remaining amine was determined on guinea-pig intestine. In the test kept at + 4° C there was found $0.620\gamma \pm 0.010$ and in the 37° C one there was $0.612\gamma \pm 0.010$. The difference observed, 0.008 ± 0.014, is not statistically significant, and shows that a possible spontaneous inactivation of histamine under the specified conditions is not of such a magnitude that it makes itself apparent even with a very accurate biological determination.

Next it seemed to be necessary to examine whether the histamine inactivation observed might be caused by bacteria, since it has been known for a long time that histamine does not keep in solutions infected with bacteria. WERLE (1940 b, 1941) as well as BUCHERER & ENDERS (1942) have found inter alia that bact. fluorescens, pyocyaneum, coli and proteus have histaminolytic effect. In order to illustrate this question of bacterial inactiva-

tion I have examined the inactivation of histamine in a water solution containing inter alia bact. pyocyaneus and coli. It proved that this was most strongly pronounced at pH 5 (fig. 11A). The histaminolytic effect decreased rapidly at pH 6.6 (fig. 11B).

All the glassware used for the examination of the histaminolytic power of the plasma was most carefully cleaned and dried as a rule in a temperature of 40 to 60° C for a couple of hours but they were not actually sterilised. There was, however, no reason to assume that there was any great bacterial impurity in the preparations, and I have not closely analysed the question of the bacterial histamine inactivation in my tests.

In 7 tests from men and non-pregnant women I found that the inactivation was only about half as great after adding octyl alcohol or toluol. Whether this decrease in the inactivation can be attributed to the ability of the chemical agents to check the growth of the histaminolytic bacteria, or whether the chemicals have a destructive effect on the histamine inactivating enzyme to be found in plasma and thus decrease the cleavage of the histamine, I have not investigated. I must therefore leave the question open for the present as to whether and to what extent the histaminolytic power observed from men and non-pregnant women is to be attributed to the effect of bacteria or to that of a histaminolytic enzyme or to both.

It is quite another thing when examining blood with a stronger histaminolytic power, there being a far stronger inactivation in a considerably shorter time of incubation. If any of the histaminolytic power in non-pregnant subjects should be caused by the effect of bacteria, the relative increase in this power observed in pregnant women (see page 66) will thus be of a still greater magnitude than that described. The part played by bacteria in producing the strong histaminolytic power observed in pregnant women seems to be of no importance.[1]

---

[1] In 12 tests taken from women with lues I or II, I have found an average histaminolytic power of 0.008 γ/ml/h, ± 0.001 ($\sigma = 0.004$, max. 0.015 γ/ml/h, min. 0.002 γ/ml/h), in 3 women with asthma bronchiale 0.007, 0.010 and 0.011 γ/ml/h. These values may possibly be regarded as significant of an increased histaminolytic power in these diseases; a more detailed analysis of this is planned. In 3 cases of peptic ulcer, in 2 cases of nephritis and in 1 case of acanthosis nigricans I have found entirely normal values. For blood tests from luetici I am indebted to Dr. B. TARRAS-WAHLBERG, Chief of the Department for Skin and Venereal Diseases at St. Göran's Hospital, and for the other blood tests from pathological cases to Professor H. BERGLUND, Chief of Medical Department IV, St. Erik's Hospital.

B. Pregnancy and Puerperium.

Healthy subjects.

As has been already mentioned a number of authors have studied the histaminolytic power of the blood during pregnancy. MARCOU et al. (1938) were the first to give information about a strong histaminolytic effect in the blood from pregnant women. They observed that it was particularly noticeable immediately before parturition, when according to their method the histaminolytic power rose to about three times as much as was to be observed in non-pregnant subjects. As an example of the variation among pregnant women the authors indicated that the histaminolytic effect could vary from an inactivation of 60 to 95 % of added histamine.

After re-examining the results of MARCOU et al. WERLE & EFFKEMANN (1940 a) found that already in the third month of pregnancy the histaminolytic effect was $3^1/_2$ times greater than normally. During the sixth and seventh months the enzyme effect had reached its climax — it corresponded then to between 20 and 25 times the normal — and after this period it sank slowly to about $^2/_3$ of maximum values. After parturition the histaminolytic effect of the blood disappeared rapidly, after only nine days there was nothing to be found. The authors' cases given in tabular form show that the values of different pregnant subjects during one and the same month vary considerably.

Using the WARBURG method ZELLER & BIRKHÄUSER (1940) showed an increase in the diamine oxidase activity during pregnancy that was twenty times as great or even more as compared with the normal. From extensive clinic material LABHARDT found the oxygen uptake to exceed the values observed in non-pregnant women by at least 25 % from the fourth month. In several cases, however, the author found such values as early as in the second month of pregnancy. The diamine oxidase diminishes in the blood during the actual parturition and only six days after the confinement there is not much more than is the case in non-pregnant subjects.

In a later work ZELLER (1941 e) related the enzyme effect to the months of pregnancy and found that the oxygen consumption rose from the third to the seventh month, then reaching

values about 20 times greater than the normal. During the 8th and 9th months the enzyme effect is somewhat weaker, and during the last it rises again to the values of the seventh month. At parturition the enzyme effect sinks somewhat, and by the fourth day it is again at a normal level.

ZELLER (1941 e) found an increased quantity of enzyme in the blood from the third to the seventh months of pregnancy by means of the indigo disulphonate reaction. During the seventh month the activity sank to rise again during the tenth. At the time of parturition the values were somewhat lower. The decrease then continued rapidly, and by the fourth day after parturition no definite increase in the values could be observed. The author pointed out, that the discoloration of the indigo disulphonate in different sera is in proportion to the oxygen consumption which he registered at the same time.

By means of their modified indigo sulphonate method NEUMANN & EBBINGE (1942) found that serum from non-pregnant women in very exceptional cases discoloured indigo sulphonate. They found, however, that in certain cases pregnant subjects cause a decided discoloration already in the second month, but at the same time they say that they have obtained uncertain reactions even in the fifth. Out of 32 women examined in the second to the fifth months, 5 showed negative or doubtful reactions. During the latter half of pregnancy, however, only 4 out of 90 showed no positive reaction. The authors' reaction was not quantitative. It was negative on the second day after parturition or earlier. Only in one case did they find a positive reaction on the nineteenth day after parturition.

With the determination method by means of ammonia formation ZELLER (1940 b, 1941 c) found that there was an increase at least 50—60 times during pregnancy.

If the results of the experience gained as to the histaminolytic power of the blood during pregnancy be summed up, we shall find that it seems to rise above the value occasionally observed in non-pregnant subjects during the second, third or fourth months of the pregnancy and that the enzyme effect subsequently rises to its maximum during the fifth to seventh months. During this period the enzyme effect seems to rise 30—50 times the level observed in non-pregnant women. After this it sinks, once again to reach a possible maximum during the last month. At the

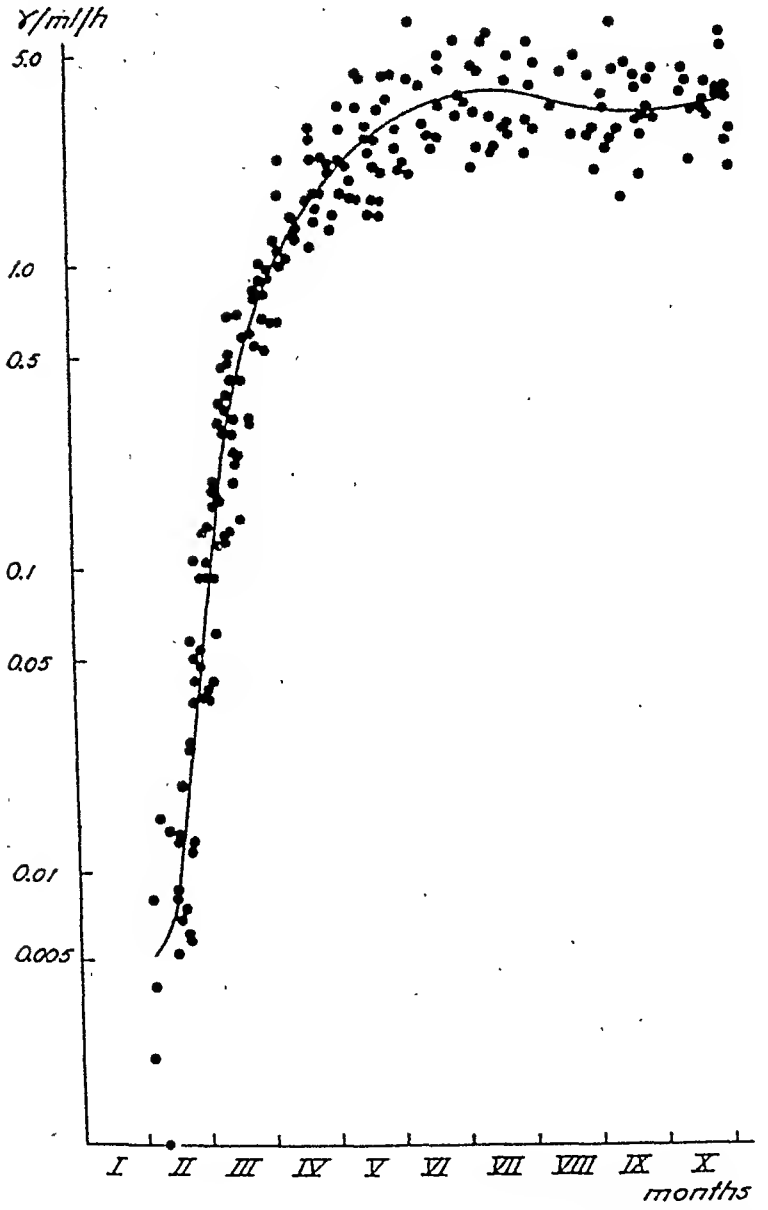


Fig. 12. Histaminolytic power of plasma from ca. 45 healthy pregnant women.
Abscissa: months of pregnancy calculated from first day of last menstruation.
Ordinate: histaminolytic power expressed in γ inactivated histamine per ml plasma per hour.

time of parturition a decrease seems to take place in the enzyme effect, which 2—6 days after the confinement does not seem to be higher than normal.

As has been earlier pointed out the investigations of the histaminolytic power of the blood have been carried out under ex-

tremely varying conditions, which may explain the different results. It is furthermore stated that the investigations do not give any sufficiently accurate conception of the physiological variation of the histaminolytic principle in pregnant women.

Own results.

I have examined the histaminolytic power of the blood in healthy pregnant women according to the method above described (page 43). The test subjects, from 20 to 40 years of age, were healthy people under control at the Mothers' Welfare Centre, St. Erik's Hospital, and at the National Organization for Sexual Enlightenment, staff members of the Stockholms Sjukhem and of the Pharmacological Laboratory, Stockholm and personal friends.

In this chapter we shall look more closely into the histaminolytic power during various periods of pregnancy, but I should first like to give a complete picture of the variations of this power during the whole period of pregnancy. Fig. 12 shows the majority of the blood analyses made from healthy pregnant subjects, ca. 200 tests from ca. 45 subjects. As will be seen in fig. 12 the histaminolytic power — as it is expressed in this work — in women during different phases of pregnancy may rise to over 1,000 times. In order to be able to give a surveyable picture of the enzyme activity during the whole course of the pregnancy on one single curve a logarithmic scale has been adopted for the histaminolytic power.

The characteristic variations in the activity of the histaminolytic power will now be analysed more closely.

The histaminolytic power during the first four months of pregnancy.

On an average I have found a histaminolytic power going up to 0.005 γ/ml/h in non-pregnant women. It is difficult to say when the increase actually begins to take place, but in three tests from subjects in the fifth week of pregnancy I have found values of 0,002, 0,004 and 0,008 γ/ml/h, and in three tests from

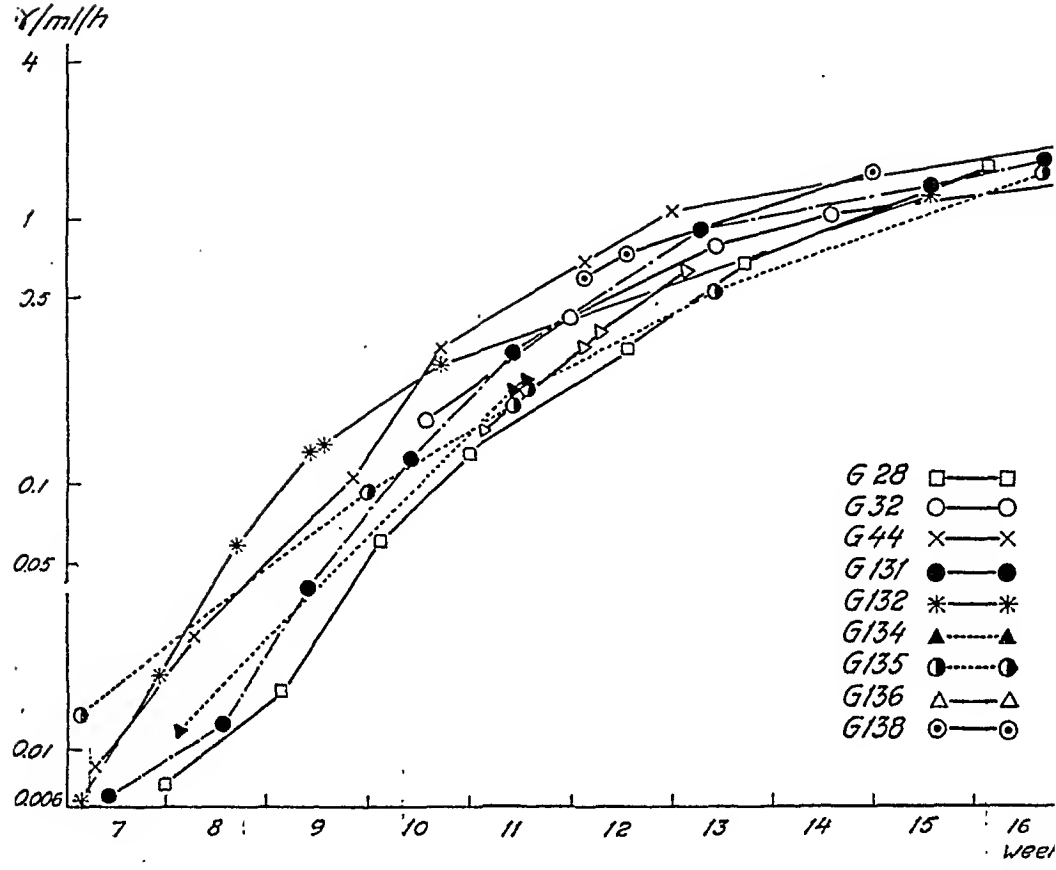


Fig. 13. Histaminolytic power of plasma from 7th to 16th week of pregnancy in nine healthy women.[1]

Abscissa: weeks of pregnancy calculated from first day of last menstruation. Ordinate: histaminolytic power.

the sixth 0,001, 0,013 and 0,015 γ/ml/h. The values obtained indicate the likelihood of there being an increased histaminolytic power during the sixth week, but the material at my disposal is too little to establish this. It is not until the seventh week that a definite increase is obvious. In 8 plasma tests taken from this week I have found an average histaminolytic power of 0.010 γ/ml/h (max. 0.019, min. 0.006 γ/ml/h).

During the seventh to the sixteenth week I have followed the histaminolytic power of plasma by taking repeated tests from nine healthy women, and the results are to be found

[1] Reading the proof the author wishes to add the two following cases. G 140: 73rd day of pregnancy = 0.30 γ/ml/h, 78th day = 0.44 γ/ml/h. Case G 141: 86th day = 0.32 γ/ml/h, 93rd day = 0.54 γ/ml/h. These cases further confirm the conclusions in the text concerning the accuracy with which pregnancy can be calculated by means of the determination of the histaminolytic power in plasma.

in fig. 13. From this it will be seen that an increase takes place from ca. 0.01 to ca. 1.5 γ/ml/h of this power during this period. During the ninth to the eleventh week there is a daily average increase of between 10—15 %, and as I have determined the histaminolytic power in this work with an accuracy of ca. 7 %, it should be possible to observe the increase from day to day.

In order to prove the correctness of this hypothesis I have examined the histaminolytic power of blood from four healthy subjects for two consecutive days. The blood tests were taken during the ninth to the twelfth week of pregnancy, and the results are recorded in table 7. There is also a graphic registration of all the cases in fig. 13.

Table 7.

Increase in histaminolytic power of plasma from one day to the other estimated on 4 women during third and fourth months of pregnancy.

Case	Day of pregnancy	Histaminolytic power γ/ml/h	Day of pregnancy	Histaminolytic power γ/ml/h	Increase %
G 132	59	0.131	60	0.136	4
G 135	73	0.188	74	0.237	26
G 134	73	0.218	74	0.237	9
G 136	78	0.323	79	0.354	10

As will be seen from the values in table 7. all the cases show an increase in the histaminolytic power from one day to the next, the average being 12 %, and therefore these observations seem in good agreement with what was to be expected. The variation in the values is to be explained by the errors of the method as well as by the individual variations.

Fig. 13 also shows that the values for the histaminolytic power in different women seem to be well related to the time of pregnancy. For instance, a value of 0.18 γ/ml/h seems only to be found in women between the 63rd and 75th days after the first day of the last menstruation, and from this it is clear that from about the 8th to the 13th week of pregnancy it is possible with the help of the histaminolytic power of plasma to establish with great accuracy how far the pregnancy has proceeded in a healthy subject. Judging by the material so far obtained, it seems that on the basis of the knowledge of the histaminolytic power in a healthy subject it is possible to determine in what week of pregnancy she

is as near as to within about 6 days in two cases out of three (calculated from the values in figs. 12 and 13).

Fig. 13 shows still further that the increase in the histaminolytic power during the third and fourth months proceeds with a strikingly constant percental rate in all women, and this seems to be the case whether the initial increase begins early or late. Thus we find that the curves for both G 132 and G 28 run fairly parallel in spite of the fact that in the eighth week G 132 had a histaminolytic power which was about 4 times greater than G 28.

The histaminolytic power during the last six months of pregnancy.

During the time after the fourth month and up to parturition we find according to fig. 12 a maximum of histaminolytic power in the 6th—7th months, after which the effect decreases somewhat to rise again during the last month of pregnancy. In the individual curves from four healthy pregnant subjects shown in fig. 14 there are also tendencies to maxima and minima. Thus case G 131 shows a pronounced maximum in the 22nd week of pregnancy, after which the histaminolytic power gradually decreases. In this case, however, I have not been able to observe any rise towards the end of the pregnancy. In case G 32 there seems to be a slightly pronounced maximum in the sixth month, a minimum in the eighth and subsequently increased values.

Cases G 132 and G 47 are of particular interest. I was able to follow the plasma values of the former during two successive pregnancies. The values from the first are indicated by means of circles (o) in the figure, those from the second by dots (●). After the figure had been handed in for printing I received yet another value, 2.9 γ/ml/h in the 39th week of pregnancy of the latter. This value, like that indicated in the curve at the end of the 36th week, is practically on the same level as those from the former pregnancy. The question now arises whether this agreement in histaminolytic power during two pregnancies is a regular occurrence or whether it is simply chance that has caused it. I have not carried out any more investigations from different pregnancies from one and the same woman, and I am therefore not in a position to decide this

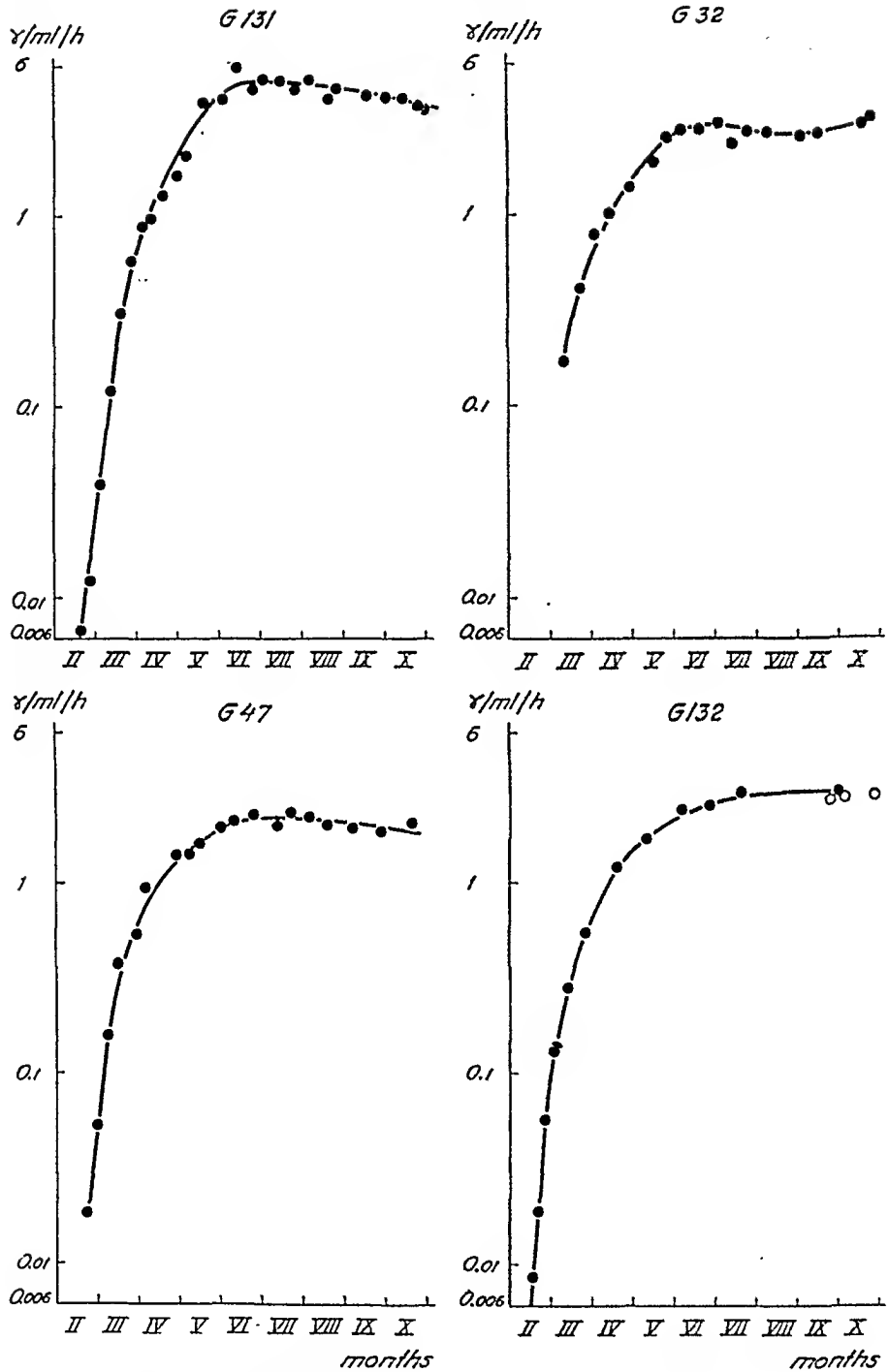


Fig. 14. Histaminolytic power of plasma in 4 healthy women.

G 132: o indicates values from an earlier pregnancy (see text!).
G 47: This woman had no menstruation for 8 months previous to beginning of pregnancy. In this case confinement was assumed to take place in 40th week.
Abscissa: months of pregnancy calculated from first day of last menstruation.
Ordinate: histaminolytic power.

question, which nevertheless seems to be of such interest that it warrants further investigation.

Case G 47 had had no menstruation for 8 months previous to becoming pregnant. Parturition had to be reckoned as taking place at the end of the 40th week. In this case, too, we find a curve completely coinciding with those seen in other pregnancies with a slightly pronounced maximum in the 6th—7th month. The last value taken on this subject before parturition indicated a slight increase of the histaminolytic power.

Fig. 14 will also serve as the illustration of the ordinary, continual change in the histaminolytic power of a healthy woman when repeated investigations are made, without there being any other occasional fluctuations than those which are due to errors in the method of determination.

Figs. 12 and 14 show the values which the histaminolytic power arrives at in plasma in different pregnant subjects. A histaminolytic power of ca. 3 γ/ml/h may be said to indicate a mean value of the plasma tests taken after the enzyme effect has reached its climax. I have not observed any value less than 1.6 or greater than 6.0 γ/ml/h in any healthy pregnant woman after the 22nd week up to the time when the labour pains begin.

Parturition.

In order to illustrate the relation of the histaminolytic power during the confinement I have carried out the following investigations. In a healthy woman, who during the last two months of pregnancy has had values varying between 3.7 and 4.6 γ/ml/h, a blood test taken immediately after the spontaneous rupture of the membranes showed a value of 3.5 γ/ml/h. In this case the rupture was the first sign observed of the confinement having begun. Another woman during the last two months of pregnancy had shown a histaminolytic power varying between 2.6 and 2.7 γ/ml/h. She felt slight pains for a couple of days before the real pains began. The rupture of the membranes then took place within $1^1/_4$ hrs and the confinement within about $3^1/_2$ hrs. A blood test was taken immediately after the rupture and showed 3.2 γ/ml/h. In the case of a third woman two tests were taken 7 and 11 hours after the pains had begun. When the first test was taken the pains were only slight, at the time of the latter they were bad. The histaminolytic power was 4.7 and 4.3 γ/ml/h

respectively. Parturition took place ca. 3 hours after the last test. In one case of premature parturition values of 9.1 and 9.3 γ/ml/h were observed in two tests taken at intervals of 5 hours during slight pains.

The above examples show that the pains do not produce any uniform change in the histaminolytic power of the plasma. In the first case a slight decrease was observed, in the second a slight increase after the pains had begun. In the cases of the third and fourth women there was no definite change in the histaminolytic power during the labour pains. Although these examples cannot be said to be sufficient to demonstrate the question as to whether the labour pains influence the histaminolytic power of blood in healthy subjects, it seems as though the conclusion may nevertheless be drawn that even if the pains cause certain changes, these are not particularly significant in any case.

It only seems possible to penetrate this question more thoroughly with a method that will allow of a determination of the histaminolytic power in capillary blood, it being difficult to take repeated venous blood tests during a confinement.

Puerperium.

According to what has already been observed the histaminolytic power of the blood sinks rapidly after parturition. Different authors have, however, given extremely divergent results and I have devoted my attention to the rate with which the enzyme effect disappears from the blood.

I have taken venous blood tests from some women both a few hours before and a few hours after parturition, and have found that the histaminolytic power is generally greater after than before. Table 8 shows some experiments, and from this it will be seen that the increase in the histaminolytic power can go up to nearly 60 %, though the average remains round about 30 %; in one case no increase was observed in the enzyme effect. After this initial increase, however, the histaminolytic power decreases rapidly. Fig. 15 shows a graph of this; the histaminolytic power reaches the values for non-pregnant women during the third week. No increased value has been observed in healthy subjects after the 14th day.

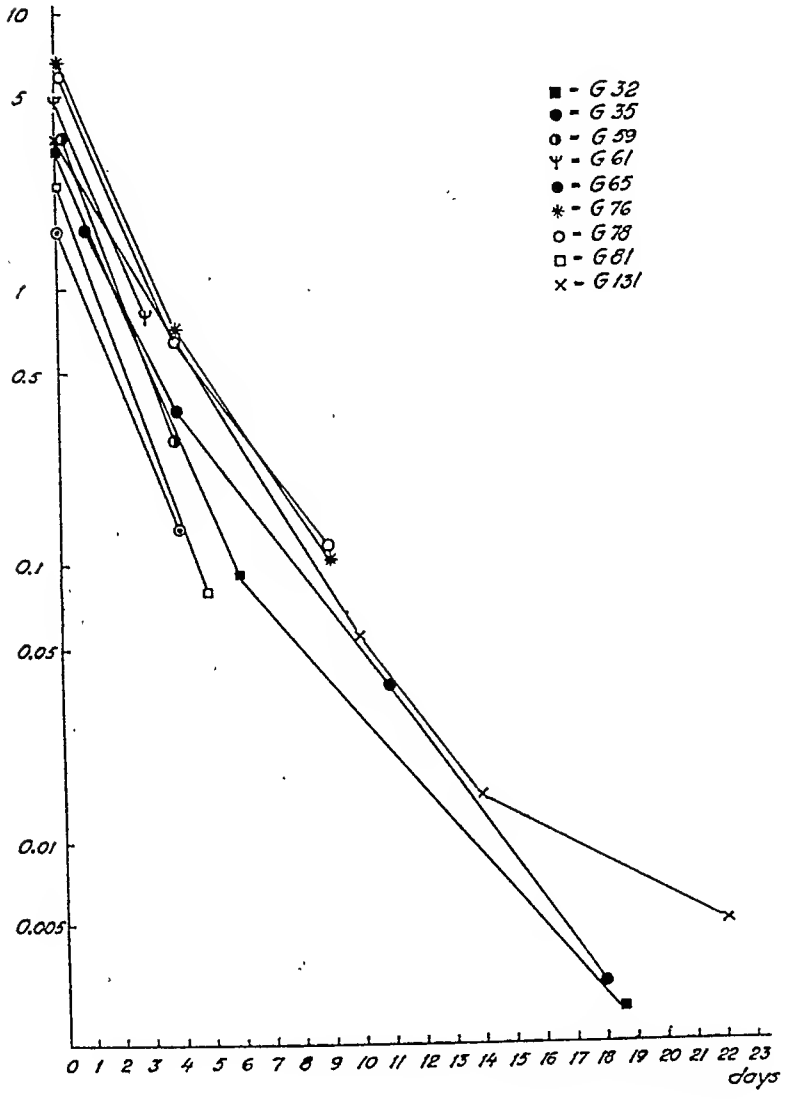


Fig. 15. Decrease of histaminolytic power of plasma after parturition.
Ordinate: histaminolytic power, γ/ml/h.
Abscissa: days after parturition.

Studying fig. 15 more closely we find that the rate of the decrease during the first few days after parturition is almost the same in the case of all women. This seems to imply — as the curve is logarithmic — that the histaminolytic power decreases with a constant fraction for the time-unit. In order to illustrate this more closely I have assumed that the decrease in the hista-

Table 8.

Histaminolytic power of plasma before and after parturition.

Case	Before parturition		After parturition		Difference	
	Hours before parturition	Histaminolytic power of plasma γ/ml/h	Hours after parturition	Histaminolytic power of plasma γ/ml/h	γ/ml/h	%
G 59	3	3.2	2	3.8	+ 0.6	+ 19
G 72	ca. 48	5.8	3	8.0	+ 2.2	+ 38
G 76	4	5.3	2	6.7	+ 1.4	+ 26
G 78	2	3.8	4	6.0	+ 2.2	+ 58
G 81	3	2.4	2	2.4	0	0

minolytic power takes place as a monomolecular reaction according to the formula

$$k = \frac{1}{t} \log \frac{C_o}{C_t},$$

where t = time, C_o = the original amount of histaminolytic power expressed in γ/ml/h and C_t = the amount subsequently remaining. In table 9 the values of the histaminolytic power and the factor k are indicated. The values given for k support the hypothesis referred to, though the material at my disposal is too little to definitely establish it. The fact that in certain blood samples C_o has been determined before, in others immediately after parturition and that the time for all the tests has not been taken more accurately than to within half a day

Table 9.

Decrease in histaminolytic power of plasma during first 3—6 days after parturition, $k = \frac{1}{t} \log \frac{C_o}{C_t}$, *see text.*

Case	C_o Histaminolytic power at parturition γ/ml/h	C_t Histaminolytic power at time t after parturition γ/ml/h	t time, days	k
G 61	4.7	0.79	3	0.26
G 65	1.6	0.13	4	0.27
G 59	3.5	0.28	4	0.26
G 78	6.0	0.61	4	0.25
G 76	6.7	0.67	4	0.25
G 81	2.4	0.079	5	0.30
G 32	3.2	0.091	6	0.26

must naturally influence the size of the constant. No consideration, however, has been taken to this when establishing k. According to the values obtained for k — 0.25 to 0.30 — a daily decrease of 44—50 % takes place in the histaminolytic power of the blood during the first six days after parturition.

In four cases, when caesarean operations interrupted the pregnancy at the end of the fifth month, I found about the same percental decrease in the histaminolytic power during the first week after the operation.[1]

Imminent abortions, extra-uterine pregnancy.

Investigations of the histaminolytic power of the blood and the diamine oxidase content in abortion were carried out by EFFKEMANN & WERLE (1940 b) and LABHARDT (1941, a, b). The former authors found that cases of imminent, incipient and incomplete abortions during the first to the fourth months of pregnancy showed as against normal pregnancies decreased histaminase equivalents in defibrinated blood. In serum the histaminase equivalents in the abortion cases were somewhat lower in relation to the normal than was the case with defibrinated blood. LABHARDT found in 9 abortions about the same values as in healthy pregnant women. After the foetus had been expelled, the diamine oxidase content sank in 24 hours to the level observed for non-pregnant subjects.

In view of the conflicting results of the earlier investigations I have carried out some investigations on women with uterine bleedings during the first half of their pregnancies.

The women examined have been patients at the Obstetrical Department, St. Erik's Hospital, Stockholm, or have received medical care at the Mothers' Welfare Centre at St. Erik's Hospital, Stockholm.[2]

Case G 64 (fig. 16) had been well during the earlier part of the pregnancy, but on the 103rd day after the first day of the last menstruation there was severe bleeding. On examining the histaminolytic power in a plasma test taken during the morning of

[1] The cases have been treated at the Gynecological-Obstetrical Clinic, Caroline Hospital, Stockholm, to whose chief, Prof. A. WESTMAN, I am indebted for his having placed the cases at my disposal.

[2] I am greatly indebted to Dr. S. CLASON, Senior Physician of the clinic and Dr. T. WALENTIN for rendering valuable assistance and advice.

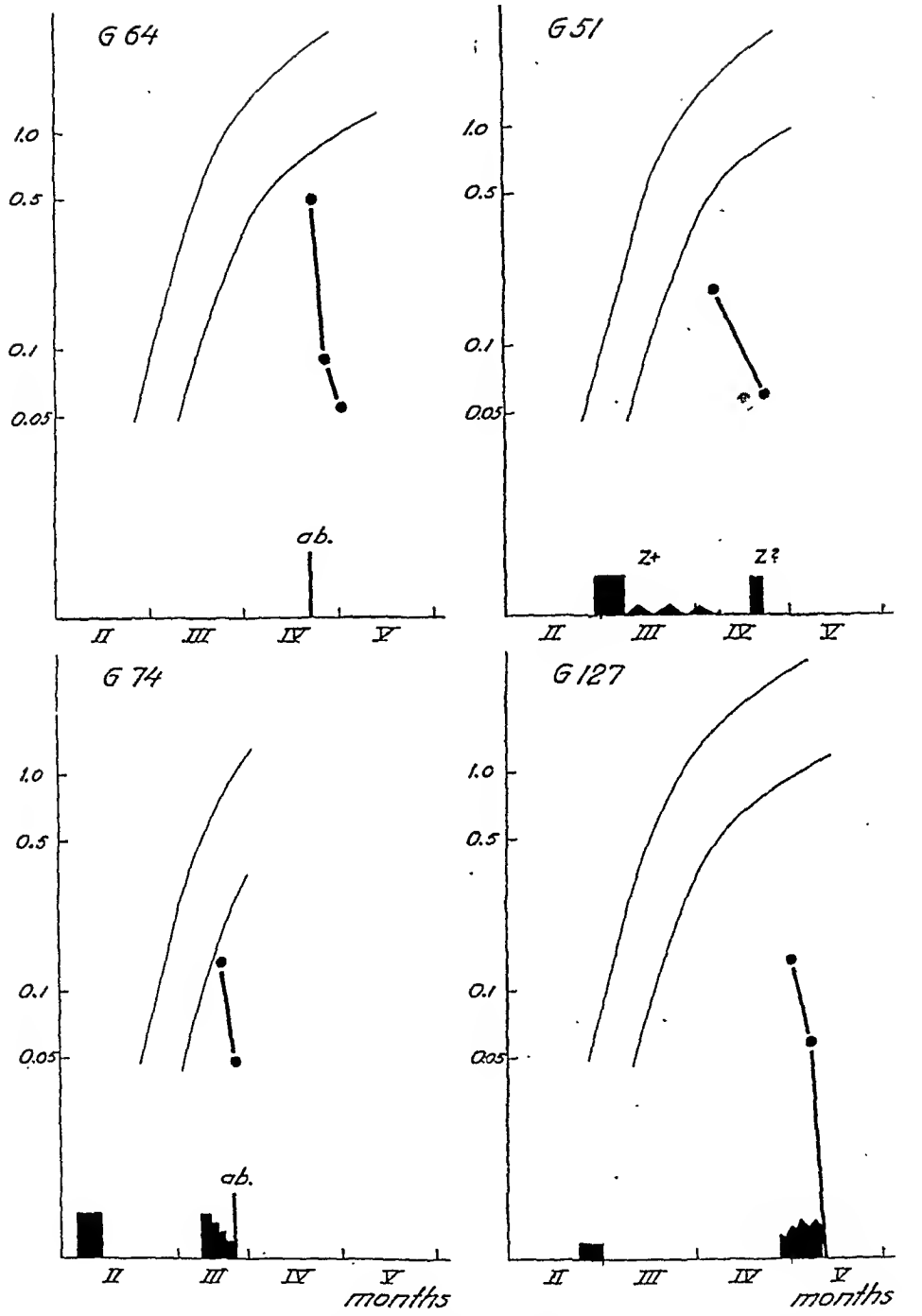


Fig. 16. Histaminolytic power of plasma in four cases of abortion.

Thin curved lines indicate limits within which are to be found ca. 19 of 20 values from healthy women.
Stems on base line indicate approximate uterine bleedings.
Z + Aschheim's-Zondek's reaction positiv.
Z? = » » » dubitative.
ab. = abortion.
Ordinate: histaminolytic power, γ/ml/h.
Abscissa: months of pregnancy.

the same day I found 0.49 γ/ml/h. Healthy women normally show about 1,3 γ/ml/h at this time of pregnancy. During the evening a defective egg was expelled. Further examinations of the plasma during the two following weeks showed a rapid decrease in the values.

Case G 51 (fig. 16). A woman who had been well during the first part of the pregnancy got a fairly bad bleeding which lasted for 10 days during the second month. After this there were slight bleedings on and off during the following month, at the end of which time the histaminolytic power of the plasma was 0.18 γ/ml/h, or about one fourth of what is to be expected in normal pregnancy. Within the course of two weeks the histaminolytic power sank considerably, and during this time the subject had a bleeding resembling an ordinary menstruation.

Case G 74 (fig. 16). At the end of the sixth and at the beginning of the seventh week of pregnancy a previously healthy woman had a fairly bad bleeding and was given progestine treatment. At the beginning of the eleventh week, however, there was another bleeding which grew less during the course of about a week. At the end of the eleventh week the histaminolytic power was 0.14 γ/ml/h. The normal value at that time is about 0.35 γ/ml/h. At the beginning of the 15th week a defective egg was expelled, and on the following day a considerable decrease was observed in the histaminolytic power (0.05 γ/ml/h).

Case G 127 (see fig. 16). During the eighth week of pregnancy a healthy woman had a slight bleeding; there was another in the middle of the sixteenth week, which gradually increased. On this latter occasion the size of the uterus corresponded to what it usually is in the third to fourth month. The patient underwent anti-abortive treatment at the hospital with daily injections of progestine and this was continued until the end of the seventeenth week. The histaminolytic power of the plasma at the end of the sixteenth week was 0.20 γ/ml/h (as against the normal 1.7 γ/ml/h at this time) and then sank to 0.07 γ/ml/h at the end of the seventeenth and continued to fall still further. The bleedings ceased in the eighteenth week, without any parts of foetus being observed, and the uterus diminished in size.

In all the four cases described there have been uterine bleedings for short or long periods, which have resulted in abortion. The values of the histaminolytic power have been considerably below

the normal, and have sunk still more as the bleedings have become less.

In Case G 64 immediately before the abortion took place a value was observed which was only one third of what was expected. In case G 74 there was a probable decrease in the histaminolytic power ere the abortion took place. Thus in both these cases it may be said that the histaminolytic power was ominous of a serious disturbance in the normal conditions. In cases G 51 and G 127 it has not been possible to establish the exact time for the expulsion of the egg. In consideration of the usual clinical observations case G 127 was not considered a definitely bad prognosis and received anti-abortive treatment. Even before it had been definitely established by the clinical examination that pregnancy had ceased, the histaminolytic power showed a strong decrease. It is probable that such a strong decrease in the histaminolytic power as was observed in this case is inconsistent with a survival of the egg. Thus this case seems to show that the knowledge of the histaminolytic power of plasma might contribute to a more accurate indication for the anti-abortive treatment.

In contrast to the four cases of uterine bleedings which ended unsatisfactorily, I should like to call attention to the following, when abortion did not take place.

Case G 66. (fig. 17). At the end of the third month of pregnancy this subject had a fairly severe bleeding which lasted for some days and which began though less severely at the beginning of the fourth month. The patient was put to bed at the hospital and treated with progestine. The histaminolytic power of the plasma showed normal values in both cases as well as a normal increase. No further bleedings occurred, and parturition took place at the end of the 40th week when a fully developed child was born.

Case G 49 (fig. 17). During the middle of the twelfth week this woman began to have bleedings, which continued on and off until the seventeenth week. The case became more complicated towards the end of the pregnancy owing to albuminuria. In the 41st week the patient gave birth to a fully developed child. The histaminolytic power of the plasma during the bleeding showed values within the limits of what can be considered normal, though the course of the curve has an appearance deviating somewhat from the normal. For instance we find that the values

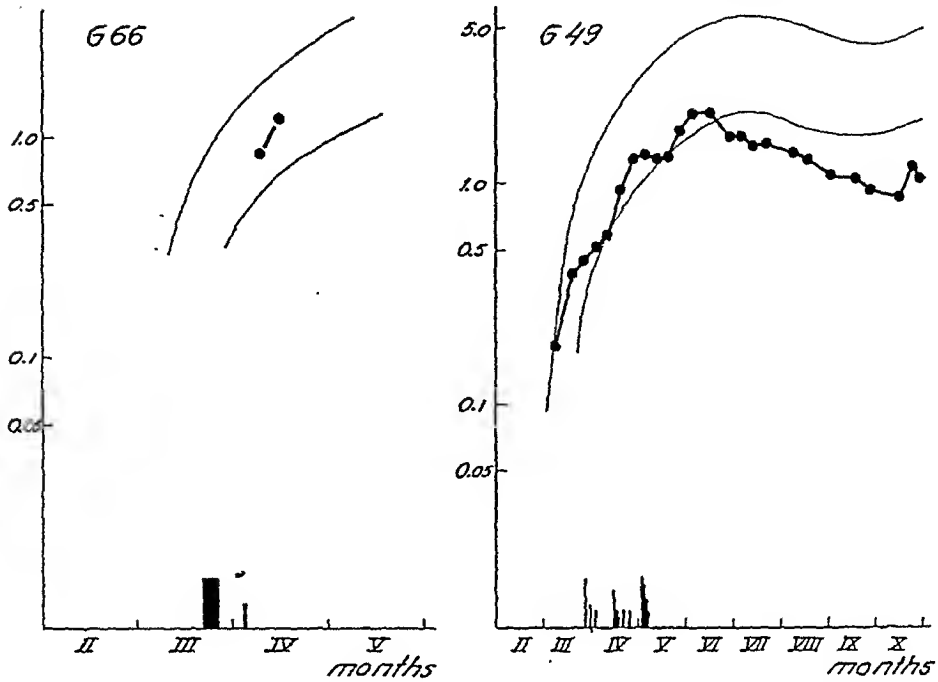


Fig. 17. Histaminolytic power in cases of imminent abortion with good prognosis.
Stems on base line indicate approximate uterine bleedings.
Thin curved lines indicate limits within which are to be found ca. 19 of 20 values from healthy pregnant women.
Case G 49 at the end of pregnancy complicated by albuminuria, see p. 85.
Ordinate: histaminolytic power, γ/ml/h.
Abscissa: months of pregnancy.

taken in the twelfth—fourteenth and the sixteenth—eighteenth weeks do not show such a strong increase in the histaminolytic power as is normally the case. This slower increase in the histaminolytic power had already made itself apparent before the bleeding had taken place. Thus I had observed the value of the histaminolytic power in the twelfth week the day before the patient got her first bleeding.

The last two cases illustrate the relation of the histaminolytic power when uterine bleedings take place that do not bring about abortion. Thus we find normal values, and possibly the otherwise regular increase of the histaminolytic power takes place with a somewhat reduced rate. This retardation of the normal increase has in one case been observed ere the bleeding had begun.

In connection with these cases it seems suitable to refer to an examination I carried out on a subject with *extra-uterine preg-*

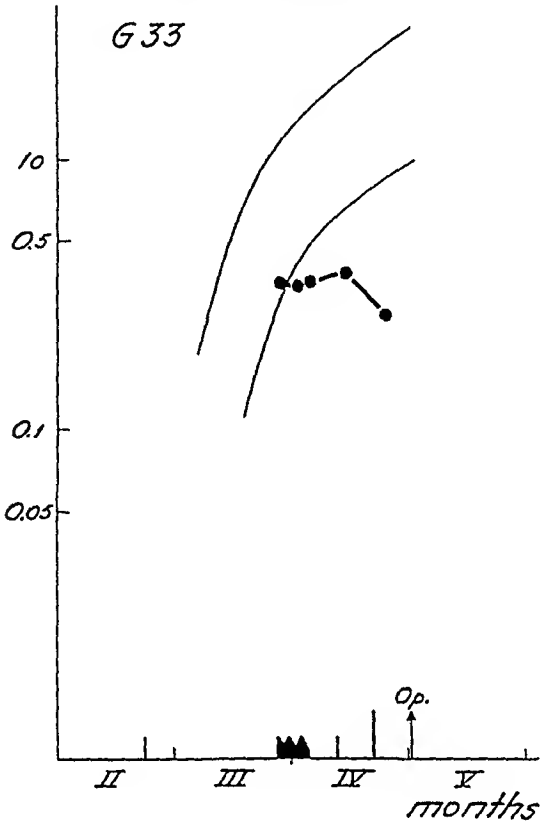


Fig. 18 Histaminolytic power in case of extra-uterine pregnancy.
Thin curved lines indicate limits within which are to be found ca. 19 of 20 values from healthy pregnant women.
Stems on base line indicate approximate uterine bleedings. For explanations see text.
Op. indicates operation.
Ordinate: histaminolytic power, γ/ml/h.
Abscissa: months of pregnancy.

nancy. WERLE & EFFKEMANN (1940 b) stated that there was no increase of the histaminolytic power in a case of extra-uterine pregnancy as compared with non-pregnant women. These authors pointed out that the histamine inactivating principle in blood and serum decreased "einige Tage nach einer zur Unterbrechung der Schwangerschaft führenden Störung" and concluded that the occurrence of an increased histaminolytic power as opposed to non-pregnant women is an indication of an *intact* pregnancy. It is, however, proved by the cases in this chapter that their conclusion cannot be correct. — According to LABHARDT (1941 a) the diamine oxidase in the extra-uterine pregnancy is the same as in ordinary cases.

Case G 33 (fig. 18) showed a slight bleeding in the second and also small bleedings from the end of the third to the end of the fourth months. The case was looked upon as being an imminent abortion and received anti-abortive treatment. At the beginning of the fifth month the patient underwent an operation for extra-uterine pregnancy perforating to the abdominal cavity. The histaminolytic power of the plasma at the end of the third month was almost bordering on the normal. No increase of this power was observed. However, afterwards, there was instead at the beginning of the sixteenth week a decrease of almost 50 % of the histaminolytic power. Six days after the last blood test was taken, the perforation took place. In this case the decrease in the histaminolytic power gave warning of the unsatisfactory development of the pregnancy.

Albuminuria and toxaemia of pregnancy.

Several authors have stressed the importance of the metabolism of histamine when intoxications occur during pregnancy, and histamine has been looked upon as being one of the causes (see FELDBERG & SCHILF, 1930). WESTBERG (1941) first and then KAPELLER-ADLER (1941) and KAPELLER-ADLER & ADLER (1942, 1943) found that during pregnancy normally occurring histidinuria decreases or disappears in severe cases of toxaemia of pregnancy and the latter authors showed that histamine appears in the urine in these pathological pictures. These authors assumed that the histaminase metabolism of the organism was disturbed in cases of toxaemia and referred inter alia to a statement by ZELLER (1941) according to which histaminase occurs in smaller quantities than is normal in patients with hyperemesis gravidarum. WERLE & EFFKEMANN (1940 b) also stated that the histaminolytic power in cases of toxaemia was decreased in comparison with that of healthy pregnant women. Two years later, however, they (1942) examined 12 cases of toxaemia of pregnancy and in none of these were they able to establish anything deviating from the normal pregnancy.

As it seems obvious, however, that disturbances in the metabolism of histamine do occur in toxaemia of pregnancy, and as the part played by the histaminolytic power does not seem to have been made clear in this connection, I have carried out

Table 10.

Histaminolytic power in 21 women with pregnancy albuminuria and toxaemia.

Case	Oedema	Hypertonia	Eclamptic symptoms	Albuminuria	Parturition in week of pregnancy	Foetus: Weight g: Surviving	Foetus: Weight g: Still born or surviving only a few hours	Foetus: Death	Histaminolytic power of plasma at stated weeks γ/ml/h
Histaminolytic power less than normal.									
G 93	+++	++	—	+++	39	3050			37—39 w.: 0.67; 0.67; 0.79; 0.97 see fig. 19
G 49	++	—	—	+	41	3350			40 w.: 1.2; 1.1 see fig. 17
G 110	—	—	+++	+	42		3700	a. p.	42 w.: 1.1
G 80	—	+	—	+++	39		1880	a. p.	26 w.: 1.2
G 65	—	++	+	++	40	2270			36—40 w.: 1.3; 1.1; 1.1; 1.6
G 106	+	+	—	++	42	2720			39—40 w.: 1.2 1.2; 1.5; 2.0
Normal histaminolytic power.									
G 69	+	+++	++	++	33		1750	s. p.	Day of part.: 2.0
G 75	—	++	—	+	36		1760	a. p.	35 w.: 2.0
G 70	+	—	+	+	30			a. p.	22—30 w.: 1.6—2.3, see fig. 19
G 89	+	—	+	++	35		2100	a. p.	34—35 w.: 3.0—3.1, see fig. 19
G 126	—	—	—	+					34—35 w.: 3.4; 2.5; 3.4
G 87	+	+++	+++	+++	ca. 39	3020			Day of part. 3.5
G 97	—	—	—	+	40	3580			39—40 w.: 3.8; 4.5
Histaminolytic power greater than normal.									
G 139	+	+	+	++	38	1880			Day of part. 4.7
G 91	+	+	+	+	ca. 40	3070			ca. 39—ca. 40 w.: 5.0; 5.2; 5.8
G 30	+	+	+	+	38	1900 2530			33 w.: 6.0
G 137	(+)	+	—	+	40	2420			36—40 w.: 3.8; 6.1; 6.1; 6.9, see fig. 19
G 88	++	++	++	+	35		1460	p. p.	34 w.: 7.2; 6.7
G 99	++	++	++	+++	44	3850			38—40 w.: 5.8; 5.9; 5.3; 6.5
G 90	(+)	++	—	+	ca. 39		3060 2360	a. p.	ca. 38—ca. 39 w. 8.3; 7.8; 8.6
G 96	+	++(+)	+	++	37	2500			30—37 w.: 5.6—7.9, see fig. 19

some experiments on the histaminolytic power of plasma in such cases.

In about 20 cases of pregnancy albuminuria which have been treated at St. Erik's Hospital, Stockholm, I have examined the histaminolytic power of the plasma on one or several occasions. The symptoms are given schematically in table 10, where there will also be found the weight of the foetus, its viability and the histaminolytic power. The values obtained are also given in fig. 19.

The cases in the table have been arranged according to the magnitude of the histaminolytic power. They have also been divided into three groups with low, normal and high values. As boundaries for the normal values I have made use of the limits within which about 19 of 20 values of healthy women are to be found.

The table shows that a considerable number of the women with pregnancy toxaemia have values deviating from the normal. Only about a third have normal, while a third have abnormally high and a third show values which are too low. In this material there is no correlation between the degree of the symptoms and the histaminolytic power. Thus we find women with different degrees of different symptoms with low, normal and high

---

Symptoms classified according to following bases of division:

Oedema

—: no oedema
+: slight oedema in hands and feet but not in face
++: obvious pre-tibial and malleolar oedema, slight oedema in face.
+++: pronounced pre-tibial, malleolar and facial oedema

Hypertonia

—: blood pressure not exceeding 140 mm Hg on any occasion
+: values between 140 and 170 mm Hg observed several times
++: values between 170 and 200 mm Hg observed several times
+++: values exceeding 200 mm Hg observed several times

Eclamptic symptoms

—: no eclamptic symptoms
+: slight occasional headache
++: pronounced headache for one week at least
+++: eclamptic attacks

Albuminuria

+: albuminuria not exceeding 2 ‰ (according to ESBACH's method of determination) observed on one or several occasions
++: albuminuria amounting to between 2 and 4 ‰ observed on several occasions
+++: albuminuria exceeding 4 ‰ observed on several occasions

a. p., s. p. and p. p. signify that the foetus died before, during or immediately after parturation.

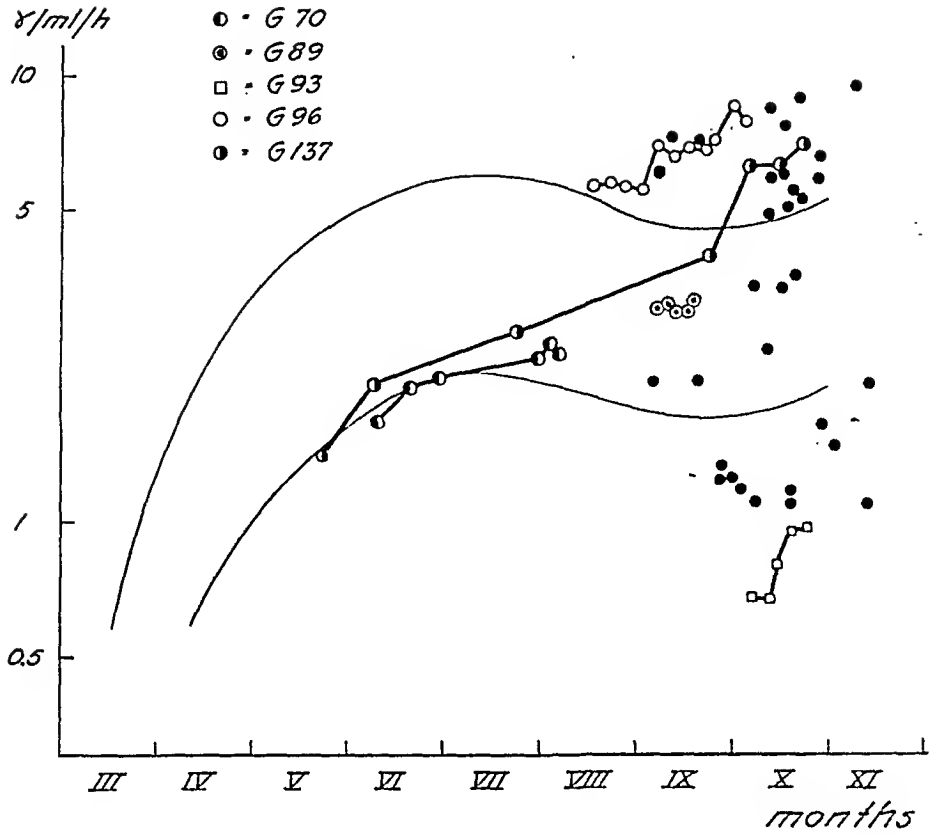


Fig. 19. Histaminolytic power of plasma in cases of albuminuria and toxaemia of pregnancy. (see table 10).

Thin curved lines indicate limits within which are to be found ca. 19 of 20 values from healthy pregnant women.
● = samples from women from whom only occasional tests have been taken.
◐ ⊙ □ ○ ◑ = samples from women from whom repeated tests have been taken.
Abscissa: months of pregnancy.
Ordinate: histaminolytic power.

values of the histaminolytic power. Nor does the length of the pregnancy nor weight and degree of development of the foetus seem to have any definite connection with this power. The material examined, however, is too little to definitely exclude the existence of any correlation between the symptoms and the absolute values.

Two of the cases mentioned deserve special attention. Case G 137, the curve of which we find in fig. 19, showed albuminuria for the first time during the 36th week of pregnancy; it is

seen that there was a definite increase in the histaminolytic power at about this time and this fact therefore seems to indicate that there may be some sort of connection between the appearance of the albuminuria and the histaminolytic power. Moreover, I should now perhaps point out that the curve for the histaminolytic power of healthy pregnant women runs very evenly at this period, and in no case of a healthy woman have I observed such a fluctuation in the histaminolytic power as in the case of G 137 (see fig. 14, page 70).

In case G 49 the histaminolytic power gradually decreased from the seventh month, so that at the beginning of the tenth it was only about $^1/_3$ of what it is normally. Slight oedemata began to make their appearance at this time, which increased considerably during the following week. In the middle of the 39th week albuminuria was observed for the first time, and from these cases it seems as though we may draw the conclusion that an abnormal curve for the histaminolytic power may anticipate something pathological (fig. 17, page 79).

The investigations so far carried out show that the appearance of albuminuria in pregnant women is often accompanied by disturbances in the histaminolytic power. The results of the experiments warrant further investigations in order to explain more fully the nature of this fact.

Premature parturition, hydatiform mole.

In two cases of premature parturition I have found increased values as opposed to the normal. Thus in one case of parturition in the seventh month I found 5.8 γ/ml/h and in another case of parturition in the eighth month 9.1 and 9.3 γ/ml/h. Further investigations are necessary to decide whether the histaminolytic power is always increased in the event of premature parturition.

LABHARDT (1941 b) observed increased values of the diamine oxidase in the case of hydatiform mole. I have been able to verify this statement in three cases. One was followed for 20 days subsequent to the mole being expelled. Blood tests taken 1, 4, 11, 18 and 20 days after the expulsion showed a histaminolytic power of 0.15, 0.15, 0.10, 0.08 and 0.06 γ/ml/hour. The values show that the

decrease of the histaminolytic power in this case took place more slowly than after a confinement (cp. fig. 15). More mola fragments were expelled on the 17th and 18th days, from which fact it seems as though the knowledge of the histaminolytic power might contribute to forming a judgment of this disease. A detailed description of this case will be published later by Dr T. WALENTIN, St. Erik's Hospital, Stockholm.

CHAPTER VI.

The histaminolytic power of placenta and its relation to that of plasma.

There have been no investigations as to the source of the histaminolytic enzyme existing in the blood during pregnancy. Several facts, however, indicate that the placenta plays a great part in this connection.

A number of investigations have shown that this organ has a strong histaminolytic effect. MARCOU et al. (1938) showed that the histamine content is higher in the blood in the umbilical artery than in the vein, and they also indicated that a thermolabile, histaminolytic enzyme existed in placenta. ZELLER, SCHÄR & STAEHLIN (1939) made a careful investigation of the histaminolytic effect of placenta and were able to state that it was due to the effect of diamine oxidase. ZELLER, BIRKHÄUSER, MISLIN & WENK (1939) found that the placenta is one of the organs containing most diamine oxidase and they presumed that the placental diamine oxidase bore some relation to the histaminolytic power of blood observed by MARCOU et al. just before parturition. As early as in 1937, however, DANFORTH & GOSHAM had shown histaminase in the placenta, and DANFORTH (1939) indicated a certain relation between the enzyme content and the efficiency of uterine contractions. EFFKEMANN & WERLE (1940 a) also showed that placenta contains histaminase; they considered, however, that it was impossible that the placental histaminase could be able to pass over to the mother's blood.

ANREP, BARSOUM, IBRAHIM & AMIN (1941) pointed out that the histaminolytic power of blood "is increased as a result of the reaction of the maternal organism to the presence of the fetus", and that "it should be expected that the increase in the histaminolytic product in the blood would bear some relation to the production of gonadotropic or estrogenic compounds".

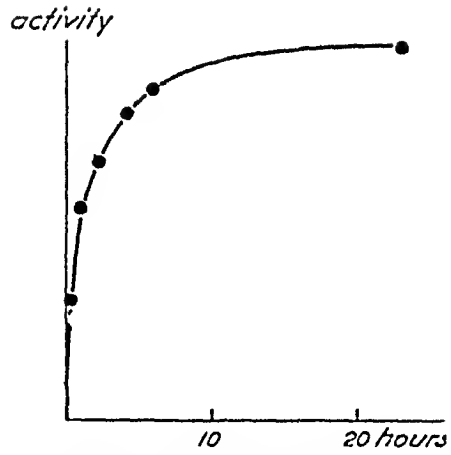


Fig. 20. Extraction of histamine inactivating enzyme from ground placenta. Extraction performed by six consecutive quantities of physiological saline.

Abscissa: time of extraction.
Ordinate: activity extracted.

Earlier investigations have shown that in the blood of pregnant women and in the placenta there is a histamine destroying enzyme. As it seems to be of interest to illustrate if there is any relation between these enzymes, I have undertaken some investigations of the kind and the degree of the histaminolytic power of the placenta.

Own investigations.

The histaminase effect of the placenta has earlier been determined in water or acetone extract of the organ by measuring the oxygen uptake or the ammonia formed or by following the histamine inactivation on guinea-pig intestine. I have found the following method suitable for testing the histaminolytic power.

Method of extraction.

The extraction of the enzyme from placenta took place in the manner now to be described. The organ underwent treatment as soon as possible after the confinement. It was dried on filter paper and thus freed from superficial blood. Membranes and the umbilical cord were removed, after which the organ was weighed. It was then ground in a mincing machine and ca. 70 g of the carefully mixed pulp was centrifuged for about 15 minutes. The fluid — which usually amounted to a few ml — was decanted and its histaminolytic power examined. After that 4 or 5 por-

tions of physiological saline, in all 180 ml, were added to 20 g of the centrifuged pulp. Before adding another portion of saline the former was removed by centrifugation. The extraction continued for 24 hours at ca. + 4° C. The different portions of saline were mixed and the histaminolytic power was determined (see below).

A considerable portion of the enzyme in the placenta is extracted by means of this method (fig. 20). It proved that physiological saline gave the best yield and a somewhat better than phosphate buffer solution at pH 7.7. A still smaller yield was obtained with phosphate buffer solution at pH 6.6 and 7.2 as well as with water. If the pulp is frozen previous to extraction the enzyme is extracted a little more quickly, but the total activity extracted is not increased by the freezing. The repetition of the extraction gives a 20 to 25 % better yield than if the whole quantity of the physiological saline is added at once. Shaking the extraction vessel does not increase the activity extracted.

Measuring the effect of the histaminolytic power.

The histaminolytic power was measured by biological determination of the quantity of histamine inactivated by the extract. The initial inactivation rate is lower with the concentration 30 γ than with 6 γ/ml. With 0.6 γ/ml the initial inactivation rate seems to be about the same as with 6 γ/ml. Fig. 21 shows that the inactivation with 30 γ and 6 γ histamine per ml takes place at a constant rate during the greater part of the reaction, while the rate gradually diminishes with an initial histamine percentage of 0.6 γ/ml. The inactivation given in fig. 22 is obtained with a constant histamine percentage and a varying quantity of extract. The ratios of the extract quantities illustrated in fig. 22 are 1 : 2 : 4. As will be seen the inactivation rates are roughly parallel to the extract content. This last mentioned experiment seems to show that a quantitative opinion of the extract content may be formed by examining the rate of the initial inactivation of the histamine. In order to be able to make comparisons possible with the histaminolytic power of the blood, investigations of this power in the placenta have been carried out with about the same initial histamine concentration as with the blood. The inactivation has been followed until about half of

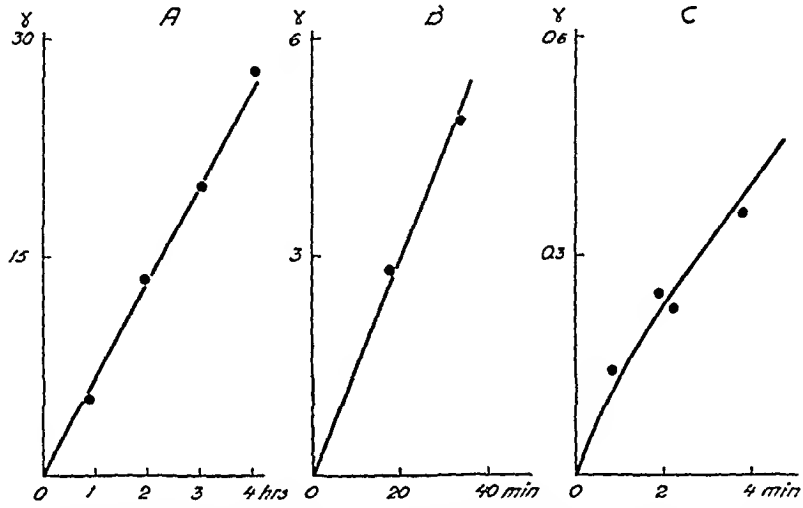


Fig. 21. Inactivation of histamine by extracts of human placenta at different initial concentrations of amine. 1 ml phosphate buffer solution at pH 6.8 mixed with 0.1 ml extract and histamine making concentration of 30 γ (fig. A). 6 γ (B) and 0.6 γ/ml (C).

Abscissa: incubation time.
Ordinate: inactivated histamine per ml reaction mixture.

the histamine has been destroyed. The histaminolytic power has been expressed in γ inactivated histamine base per ml extract per hour. The experiments have been carried out in the following manner.

The placental extract was added in increasing quantities of 0.08—0.16—0.32—0.64 and possibly 1.28 ml to a series of test

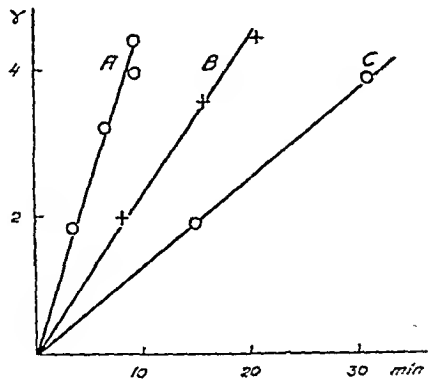


Fig. 22. Inactivation of histamine by extracts of rat placenta. Initial histamine concentration 6 γ/ml. Placental extract in A is 20 % of reaction mixture, in B 10 %, and in C 5 %. Placenta extracted with 25 volumes of phosphate buffer solution.

Abscissa: incubation time in minutes.
Ordinate: γ inactivated histamine per ml reaction mixture.

Table 11.

Prep. no.	Placental extract ml	Buffer. sol. ml	Histamine (6.08 γ/ml) γ	Incubation time, min.	Remaining histamine after incubation γ	Inactivated histamine γ	Histaminolytic power γ/ml/h
1	0.08	3.92	6.08	59.5	Not determined	—	—
2	0.16	3.84	6.08	59.5	3.65	2.43	15.3
3	0.32	3.68	6.08	60	1.35	4.73	>14.8
4	0.64	3.36	6.08	60	0	6.08	>9.5

tubes after which the volume was made up to 4 ml with m/15 phosphate buffer solution at pH 6.8. Then the tubes were incubated for a few minutes in a water-bath at 37° C, and 1 ml histamine in buffer solution containing 6.08 γ was added. After an hour's incubation the tubes were boiled up quickly in order to break off the inactivation and then kept at + 4° C until the histamine content was determined on guinea-pig intestine.

The fluid obtained when decanting after centrifuging the minced placental tissues shows a stronger histaminolytic power than the placental extract, consequently a considerably smaller quantity of this fluid must be taken for the experiment than of the extract. Thus 0.005—0.01—0.02 etc. up to 0.16 ml of the fluid was added to a series of test tubes, buffer solution and histamine being added as just mentioned. The same time of incubation was taken for both determinations.

The histamine determination was begun with the last test in every series, i. e. that with the greatest quantity of placental extract. If this contained less than half of the original quantity of histamine that was added, i. e. $< 3.04\gamma$, the penultimate preparation in the series was also tested. If this did not contain more than 3.04 γ, the procedure was continued until I came to a preparation containing more than 3.04 γ. The histaminolytic power of the placenta extract was then calculated on the basis of the test or tests showing an inactivation of between 30 and 70 % of the amine originally added. Table 11 shows an example in order to make the method clear. In preparation 4 in this example no histamine was found after incubation. In preparation 3 less than half of the amine was recovered, while in preparation 2 more than half was recovered. In this case the histaminolytic power was calculated on the basis of pre-

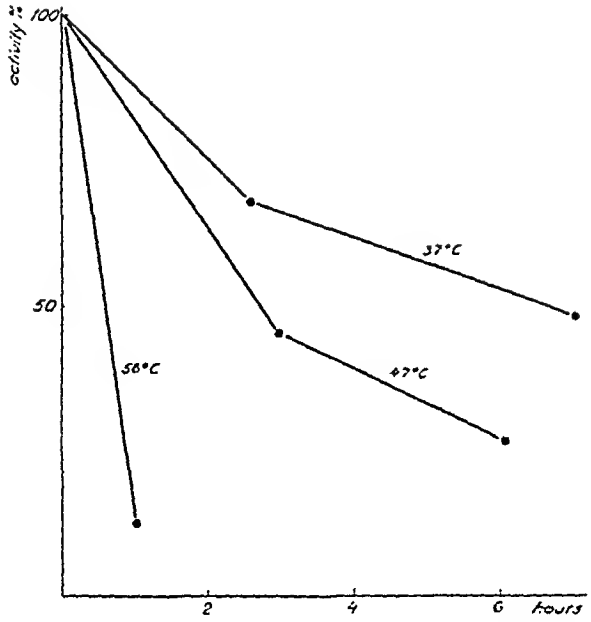


Fig. 23. Decrease of histaminolytic power in placental extracts during incubation at 37°, 47° and 56° C.

Abscissa: incubation time, hours.
Ordinate: histaminolytic power.

paration 2; 0.16 ml placental extract inactivated 2.43 γ histamine in 0.99 hours, which corresponds to a histaminolytic power of 15.3 γ per ml extract per hour.

The corresponding value for the centrifugate was added to the histaminolytic power of the extract, the activity of 1 gram of the placenta then being calculated.

When mixing equal parts of three extracts, the histaminolytic power of which was found to be 42, 14 and 8.5 γ/ml/h, it was found when examining the mixture that the activity corresponded to 21 γ/ml/h.

The histaminolytic power calculated was 21.5 γ/ml/h. The agreement can be regarded as satisfactory. Thus it seems as though this method gives a sufficiently accurate determination of the histaminolytic power in the placental extracts for the purpose of comparison.

The histamine inactivating factor is destroyed immediately at a temperature of 100° C. The effect of 37°, 47° and 56° C during different times on a water extract of placenta is shown in fig. 23. For the histaminolytic effect at various temperatures and hydrogen ions concentrations, see fig. 8 and 24. An addition of

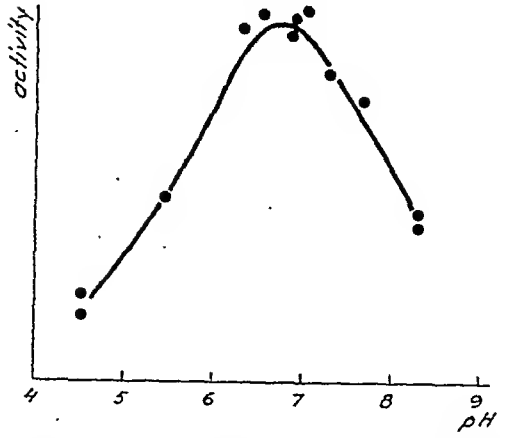


Fig. 24. Histaminolytic power of human placental extracts at different pH.

0.001 molar KCN concentration brings about a complete inhibition of the histaminolytic power, and 0.0001 molar concentration a 50 % inhibition, while KCN in 0.00001 molar concentration has no inhibiting effect whatsoever. There is a definitely noticeable inhibition with 0.0001 m phenyl hydrazine, while 0.000025 molar concentration has no such effect. Guanidine also exercises an inhibitive effect, the limit being under 0.0002 molar concentration. The inhibition with guanidine is competitive, thus in two tests with 0.0004 molar guanidine no definite inhibition could be observed at 0.0005 molar histamine, while at 0.000005 the inhibition was 76 %. When examining the distribution of the histaminolytic power in various protein fractions, 11 % of the activity was recovered in the fraction precipitated by 0.33 saturated ammonium sulphate, 31 % in the fraction 0.33—0.5 saturation and 23 % in the remaining one. 35 % of the original activity was lost. The placental extracts keep their activity almost without any loss as long as a week at + 4° C.

In the main the observations made seem to agree with the experience concerning histaminase or diamine oxidase. We also find a general agreement with the histaminolytic power of plasma when comparing corresponding properties. As opposed to the enzyme of plasma, however, the histaminolytic enzyme of placenta is partially destroyed in a water extract at 37° C in a few hours. When examining a placental extract mixed with plasma, however, I found no decrease in the histaminolytic power after incubating it for 24 hours at 37° C.

Results.

Rats.

At the time when the investigations on rats were carried out I made use of a somewhat different method from that described on page 88. Thus a phosphate buffer solution at pH 6.8 was used as extraction fluid instead of physiological saline, and the organ was extracted for only about one hour. This means — and it was confirmed in two tests — that only about half the activity was extracted, consequently the absolute values of the histaminolytic power of the rat placentas are not directly comparable with those corresponding to man, rabbit and guinea-pig. This method of extraction, however, plays a minor part when comparisons are being made of the placentas of rat.

The determination of the histaminolytic power in the extracts was carried out by following the inactivation of the histamine until half of the amine remained, after which the histaminolytic power was calculated on the basis of the quantity of histamine inactivated, the quantity of extract and the time for the reaction. When testing the placental extract from rat the author always made use of a constant quantity of extract (corresponding approximately to 0.005 g placenta per ml of the reaction mix-

Table 12.

Histaminolytic power in plasma and placentas of rats.

Rat no.	Weight g	Number of foetus	Average length of foetus mm	Plasma	Placentas		
				Histaminolytic power γ/ml/h	Weight g	Histaminolytic power mg/g/h	Total histaminolytic power mg/h
47	160	10	10	0.19	1.70	2.06	3.5
48	190	8	14	0.29	1.51	1.63	2.5
43	170	8	23	0.15	2.86	1.08	3.1
51	175	3	25	0.14	1.26	0.78	1.0
39 A	190	9	25	0.18	3.80	1.31	4.9
42	230	11	28	0.09	4.74	0.27	0.76
44	240	9	28	0.13	3.04	0.73	2.2
40	175	9	30	0.23	3.62	1.07	3.9
49	240	7	30	0.08	2.89	0.46	1.3
41	155	8	33	0.20	3.32	1.34	4.4
46	200	9	33	0.06	3.84	0.44	1.7
50	225	7	40	0.10	3.21	0.44	1.4

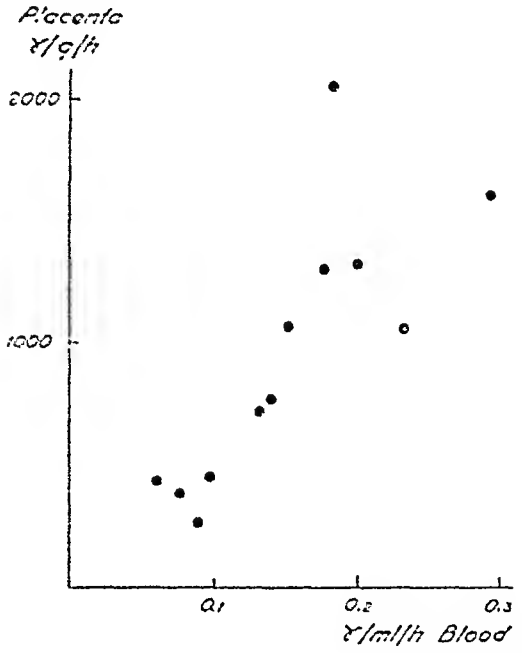


Fig. 25. Histaminolytic power of plasma (abscissa) and placenta (ordinate) of rats.

ture) and an initial histamine concentration of 6 γ/ml and the inactivation was followed by 3 or 4 tests for 1 hour.

When examining the placenta from 12 rats during the latter part of their pregnancy, I found the values of the histaminolytic power given in table 12, from which it will be seen that one gram placenta inactivates ca. 1 mg histamine per hour under the conditions stipulated. Further it seems that the histaminolytic power varies considerably with various animals, no. 47 having a 7 to 8 times stronger power than no. 42, reckoning per gr. placenta. There is no correlation with the number of foetus or their length in this material.

For the relation to the histaminolytic power of plasma observed in the same animal see fig. 25. The figure shows that the histaminolytic power of placenta and plasma are in relation to each other, inasmuch as high values of plasma are to be found in animals with high values of placenta, while low plasma values occur in animals with low placenta values.

Other animals.

Occasional examinations have been made of the placentas from rabbits, guinea-pigs and a cat. Extractions were made from guinea-

pigs and rabbits according to the method described on page 88, while the cat placenta was taken in accordance with the rat manner. The histaminolytic power calculated per gram placenta was in rabbit placenta ca. 16 γ/hour, in guinea-pig ca. 5 γ/hour and in cat ca. 0.1 γ/hour. Further investigations are necessary in order to illustrate the importance of the placenta for the histaminolytic power of blood in these animals.

Man.

In 4 healthy women I found a histaminolytic power of placenta between 56 and 102 γ/g/hour and in one case of duplex 19 γ/g/hour. No definite correlation between the histaminolytic power of placenta and that of plasma can be proved in these cases (table 13). From fig. 26 it will be seen that the power can vary considerably for one and the same blood value, and consequently it seems necessary to examine a large number of placentas from healthy women before deciding the question as to whether there is any correlation between the histaminolytic power of plasma and placenta. The determinations I have carried out on placentas from people with particularly high and particu-

Table 13.

Histaminolytic power of plasma and placenta in man.

Case	Diagnosis	Plasma		Placenta		
		Blood sample taken (a. p. = ante partum; p. p. = post partum)	Histaminolytic power γ/ml/h	Weight g	Histaminolytic power γ/g/h	Total histaminolytic power mg/h
G 47	Normal	6 d. a. p.	2.1	360	56	21
G 131	»	14 h. »	3.5	480	58	28
G 32	»	2 h. »	3.2	350	102	36
G 57	»	10 min. p. p.	2.6	475	92	44
G 133	Duplex	2 d. a. p.	1.8	740	19	14
G 49	Albuminuria	4 d. »	1.1	310	57	18
G 88	Toxaemia	5 d. »	6.8	140	134	19
G 99	»	3 d. »	6.5	640	115	74
G 96	»	2 d. »	7.9	390	168	66
G 91	»	2 d. »	5.9	390	269	105
G 93	»	1 d. »	1.0	410	62	25
G 65	»	3 h. »	1.6	400	32	13
G 90	»	2 h. »	7.4	580	154	89
G 87	»	2 h. »	3.5	520	36	19
G 72	Premature parturition	2 d. »	5.8	195	154	30

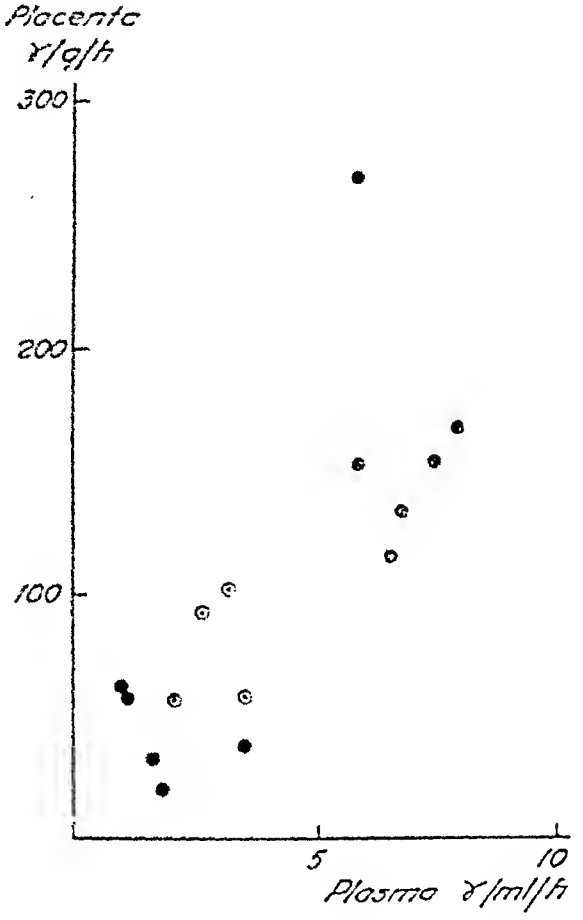


Fig. 26. Histaminolytic power of plasma and placenta in man.
Ordinate: placental histaminolytic power.
Abscissa: plasma " "
⊙ = healthy women ● = cases of albuminuria and toxaemia of pregnancy, premature parturition, duplex.
For explanations, see text.

larly low values for the histaminolytic power — i. e. cases of pregnancy toxaemia as well as one case of premature parturition — indicate that such a correlation exists. All these cases are recorded in fig. 26, from which it will be seen that all cases with plasma histaminolytic power < 5 γ/ml/hour have a histaminolytic power of placenta < 110 γ/g/hour, while all the cases with > 5 γ/ml plasma/hour have higher placental values than 110 γ/g/hour.

In some cases the placental histaminolytic power was compared with plasma tests taken a few hours before, and in others

with those taken a few days before the confinement (table 13). From fig. 19, page 84 it will be seen, however, that in cases of albuminuria and toxaemia of pregnancy the histaminolytic power of plasma appears to undergo considerable changes during the latter part of pregnancy. This fact must be partly the cause of the variation to be found in the correlation diagram (fig. 26).

The investigations of the power of placenta to inactivate histamine show that rat and man have a strongly pronouneed histaminolytie power, while it is less strongly developed in rabbit, guinea-pig and cat.

Blood investigations showed that the histaminolytic power of plasma increased strongly during pregnaney in man, less in rat and guinea-pig and not at all in rabbit.

Further investigations — first and foremost by adopting the same teehnique for all animal spceies — are necessary in order to explain the relation between the histaminolytie power of plasma and placenta. The work already carried out in this field, however, does show the existence of sueh a relation in man and rat and the histaminolytic power of plasma may to a certain extent be considered to indicate that of placenta.

CHAPTER VII.

Diagnostic and prognostic value of the histaminolytic power of plasma.

In chapter V it was shown i. a. that the histaminolytic power of plasma has a very regular course in a healthy pregnant woman. After showing a definite rise in the values round about the seventh week after the last menstruation, i. e. about five weeks after fecundation, this power then increases considerably and at about the seventh month it reaches a maximum ca. 1000 times as great as that to be found in non-pregnants. After the seventh month there is a slight decrease, but a rise is again observed somewhat towards the end of the pregnancy.

It has proved that these fluctuations in the histaminolytic power occur very regularly in a number of healthy women (fig. 13 and 14), and not in one healthy subject have I observed any important deviation from the course described. When various illnesses crop up during pregnancy, on the other hand, conditions have been different, and in this chapter I should therefore like to sum up the conclusions that may be drawn from the investigations of this power in health and disease.

The investigations in this paper show that there is no definite increase in the histaminolytic power of plasma until about the seventh week after the last menstruation. Whether any increase occurs before this time has not been decided. I must therefore leave the question as to the exact time for the present; whether this pregnancy reaction gives positive records before, simultaneously or after other pregnancy reactions, e. g. prolane excretion in the urine, must also remain unanswered. Such an excretion usually takes place before the seventh week, and in consideration of existing material I must say that ASCHHEIM's-ZONDEK's and FRIEDMANN's-SCHNEIDER's reactions are usually

positive before it is possible to show an increased histaminolytie power in plasma. This is in agreement with what WERLE & EFFKEMANN (1940 b) and LABHARDT (1941 b) noticed earlier. In order to form a definite deeision as to whether the histaminolytic power of plasma is preferable to the prolane excretion when making an early diagnosis of pregnancy, further investigations, however, are necessary with recordings of the prolanc of the urine and histaminolytie power of the plasma. Moreover it is necessary to find out whether anything else besides pregnancy can cause an inerease in the histaminolytie power of plasma (see foot note, page 62).

If the examination of the histaminolytic power in its present state does not allow of an earlier diagnosis of pregnancy than can be made with other methods, it offers instead great advantages when it concerns — so to speak — quantitative diagnosis of pregnancy round about the 3rd month. As was shown in chapter V, by examining the histaminolytie power in healthy women during this time it is possible to ascertain how far the pregnancy has gone to within ea. 6 days in 2 cases out of 3. No other clinical, ehemical or biologieal method of investigation seems to allow of such accuracy.

The importance of this quantitative diagnosis is particularly obvious when it comes to judging the time of pregnancy for women without regular menstruations previous to the beginning of the pregnaney, e. g. women with secondary or lactation amenorrhea. In ease G 47 (p. 71) there had been no menstruation for 8 months, and it was thus impossible to state when the pregnancy had begun. On the basis of the five values between 0.017 and 0.54 γ/ml/hour (see fig. 14) it was possible to predict that the eonfinement would take plaee round about July 13th 1944. The pains began on July 16th, the woman was taken into hospital on the same day, and a fully developed child was born on July 18th. 1944, i. e. only 5 days after the time expected.

If we find values of the histaminolytic power in healthy pregnant women which are not in agreement with clinical data, it is a reminder that something has occurred to disturb the physiological course of the pregnancy. I have — partly on the basis of previous experienee — shown in this paper that sueh changes take place in the ease of imminent abortion, premature parturition, albuminuria and toxaemia of pregnancy as well as in hydatiform mole. It is not elear whether other pathological pictures eause distur-

bances in the histaminolytic power, but it does not seem improbable. Thus WERLE & EFFKEMANN (1940 b) stated that they found decreased values in the case of hyperemesis gravidarum, and this I have been able to verify in one case, and I have also found deviating values in a case of pains in the abdomen with uncertain aetiology during the fourth month of pregnancy. Considering that the histaminolytic power of plasma seems to a certain extent to indicate a corresponding power in the placenta (Chap. VI), it is not improbable that diseases in the placenta might make themselves known by examining the histaminolytic power of the blood. Finally it must also be pointed out that diseases having no primary connection with the genital organs (e. g. infections) but exercising a secondary effect on the pregnancy may also influence the histaminolytic power of the blood.

Summing up it may be said that there is reason to expect that several pathological conditions may influence the histaminolytic power of plasma. The investigations so far carried out, however, have only touched upon certain of these diseases, and the following conclusions as to the diagnostic and prognostic value of the histaminolytic power must only be regarded as an example of what this reaction may be able to give.

In the case of imminent abortion occurring during the first half of pregnancy normal values of the histaminolytic power (i. e. corresponding to those found in healthy pregnants) and a normal or somewhat retarded increase of these values in repeated tests indicate a favourable prognosis. If on the contrary the plasma values sink, it seems to be a sign of an unfavourable development. Should the values neither rise nor fall during the course of some weeks, a satisfactory result may be expected (case G 49, fig. 17, p. 79) or an unsatisfactory (case G 33, fig. 18, p. 80).

It will be seen from e. g. case G 127 (fig. 16, p. 76) that the histaminolytic power may sink considerably ere the clinical examination has confirmed a bad prognosis. This observation as well as the other experiences concerning the histaminolytic power in imminent abortions indicate that repeated determinations can facilitate clinical judgment and thereby contribute to form a more rational basis of the anti-abortive treatment.

Even during the latter part of pregnancy the values of the plasma histaminolytic power may give reason to suspect that something has occurred to disturb the physiological course.

I refer to the abnormally high or abnormally low values often met with in the case of pregnancy albuminuria and toxaemia. In a couple of cases I found how abnormally deviating changes in the plasma values occurred before or simultaneously with the appearance of the symptoms. Therefore it seems not unlikely that a more extensive knowledge of the histaminolytic power in cases of pregnancy albuminuria and toxaemia might contribute to a better understanding of these diseases.

In the cases of hydatiform mole I have only a few observations, but it seems as if the histaminolytic power after the expulsion of parts of the mole decreases with a slower rate than after a parturition or an abortion. Further investigations in this field seem desirable.

Summary.

The author has determined the histaminolytic power in man and some animal species, in males and in pregnant and non-pregnant females.

After a synopsis of our knowledge of the histamine decomposing enzyme (Chap. I) and a critical analysis of earlier methods for determining the histaminolytic power of blood (Chap. II), the author gives a biological method for the quantitative determination of this power in plasma. The method is partially based on CODE's (1937) modification of BARSOUM's and GADDUM's (1935) histamine determination method. It is possible to determine the histaminolytic power with an average error of about 8 % and it is expressed as the amount of histamine base, which under certain defined conditions is inactivated by 1 ml plasma in 1 hour (γ/ml/h) (Chap. III).

Blood has been examined from a number of animal species. An increase has been proved in the histaminolytic power of blood from pregnant guinea-pigs and rats as opposed to non-pregnant animals. No such increase has been found in blood from pregnant rabbits (Chap. IV).

Plasma from 7 healthy men showed an average histaminolytic power of 0.006 γ/ml/h $\pm$ 0.001 (max. 0.016 γ/ml/h, min. 0.002 γ/ml/h) and plasma from 13 healthy non-pregnant women showed 0.005 γ/ml/h $\pm$ 0.0006 (max. 0.015 γ/ml/h, min. 0.002 γ/ml/h). The nature of the histaminolytic power in these cases is not made clear.

In pregnant women the histaminolytic power of plasma increases about a thousand times during pregnancy. The increase begins in about the seventh week after the last menstruation, i. e. ca. five weeks after the fecundation, and is particularly pronounced from the 8th to the 13th weeks, rising then 10—15 %

daily. This increase from one day to the other can be shown by the method adopted. The histaminolytic power usually reaches a maximum during the 7th month of pregnancy (ca. 3.5 γ/ml/h), then sinks somewhat and rises slightly towards the end of the time.

The increase of the histaminolytic power between the 8th and the 13th weeks proceeds so similarly in different women that in healthy subjects it is possible in 2 cases of 3 to state how far the pregnancy has gone to within ca. 6 days.

At the time of parturition the histaminolytic power increases about 30 %, and in the following week it drops ca. 45—50 % every day. No increased value has been observed in a healthy woman a fortnight after parturition.

The investigations on healthy pregnant women are based on about 200 tests on about 45 women (Chap. V.).

The histaminolytic power has been determined in some cases of imminent abortion, as well as in one case of extra-uterine pregnancy.

Cases with good prognosis showed normal or almost normal values (i. e. corresponding to those found in healthy pregnancy) while those in which the pregnancy was interrupted showed a decrease. Investigations of the histaminolytic power in cases of imminent abortion seem to be of diagnostic and prognostic value.

Cases of albuminuria and toxaemia of pregnancy show normal, increased or decreased values. In cases of hydatiform mole increased values have been observed, as opposed to those of non-pregnant women, which values have decreased subsequent to the expulsion of parts of the mole (Chap.V).

In agreement with what other authors have already shown in defibrinated blood and serum the writer has found that several characteristics of the histamine inactivating principle in plasma from pregnant women seem to be the same as those shown in histaminase or diamine oxidase (Chap. III).

A strong histaminolytic effect has been established in placenta from rats and man, while that of rabbits and guinea-pigs is weaker. In a number of cases from rats and man a certain quantitative relation has been found to exist between the histaminolytic power of plasma and placenta. The histaminolytic effect of placenta also seems to be due to the effect of histaminase or diamine oxidase (Chap. VI).

Finally the clinical significance of the histaminolytic power in the case of pregnancy has been discussed. The determination of this power can be of value when diagnosing pregnancy and establishing how far it has gone. As to disturbances in healthy pregnancies the histaminolytic power has proved useful for the diagnosis and prognosis of imminent abortions and may contribute to form a more rational basis for the therapeutics. Lastly it has been pointed out that values deviating from normal should be a warning when forming a judgment of the course of the pregnancy (Chap. VII).

I wish to acknowledge my great indebtedness to Prof. G. LILJESTRAND for his continued interest and encouragement and to my colleagues at the Pharmacological Department, Caroline Institute, for many valuable discussions.

The investigations have been supported by grants from the Swedish Society for Medical Research, from the Foundations "Therese och Johan Anderssons Minne", "P. F. Wahlbergs Donationsfond", "Torsten Amundsons fond" and from "A. B. Ferrosans stipendiefond".

References.

Ahlmark, A., Lancet, 1944, *247*, 406.

Albus, G., Klin. Wschr. 1939, 858.

Anrep, G. V., G. S. Barsoum, A. Ibrahim & A. Amin, J. Roy. Egypt. Med. Assos. 1941, *24*, 445. Quotation from Chemical Abstracts, 1943, 4782.

Anrep, G. V., G. S. Barsoum, M. Talaat & E. Wieninger, J. Physiol. 1939, *95*, 476.

Barsoum, G. S. & J. H. Gaddum, J. Physiol., 1935, *85*, 1.

Best, C. H., J. Physiol. 1929, *67*, 256.

Best, C. H. & E. W. McHenry, J. Physiol. 1930, *70*, 349.

Blaschko, contribution to a debate at XVI Internat. Physiol. Congr., Zürich, 1938. Quotation from Zeller, 1938 b.

Bonnier, G. & O. Tedin, »Biologisk variationsanalys». Stockholm 1940.

Bucherer, H. & C. Enders, Biochem. Z. 1942, *310*, 222.

Busson, B. & P. Kirschbaum, Centralbl. Bakt. u. Parasitenheilk., 1912, *65*, 507.

Code, C. F., J. Physiol., 1937, *89*, 257.

Code, C. F., C. L. Evans & R. A. Gregory, J. Physiol, 1938, *92*, 344.

Danforth, D. N., Proc. Soc. exp. Biol. 1939, *40*, 319.

Danforth, D. N. & F. Gosham, Amer. J. Physiol. 1937, *119*, 224.

Edlbacher, S. & A. Zeller, Helv. chim. Acta, 1937, *20*, 717.

Effkemann, G. & E. Werle, Arch. Gynäkol. 1940 a, *170*, 173.

Effkemann, G. & E. Werle, Zbl. Gynäkol., 1940 b, *64*, 1220.

Eichler, O. & F. Barfuss, Arch. f. exper. Path. u. Pharmakol. 1940, *195*, 245.

Ellinger, F., Arch. f. exper. Path. 1930, *149*, 343.

Emmelin, N., G. Kahlson & F. Wicksell, Acta Physiol. Scand. 1941, *2*, 123.

Feldberg, W. & E. Schilf, »Histamin, seine Pharmakologie und Bedeutung für die Humoralphysiologie», Berlin 1930.

Gebauer-Fuelnegg, E. & H. L. Alt, Proc. Soc. Exp. Biol. Med. 1932, *29*, 531.

Guggenheim, M. & W. Löffler, Biochem. Z. 1916, *72*, 325.

Kapeller-Adler, R., Biochem. J. 1941, *35*, 213.

Kapeller-Adler, R. & E. Adler, Biochem J. 1942, 36, ii.

Kapeller-Adler, R. & E. Adler, J. obstetr. gynecol. Brit. Empire 1943, *50*, 177.

Kiese, M., Biochem. Z. 1940, *305*, 22.

Kwiatkowski, H., J. Physiol. 1941, *100*, 147.

Labhardt, A., Mon.-schr. Geburtsh. u. Gynäkol. 1941 a, *112*, 1.

LABHARDT, A., Schweiz. med. Wschr. 1941 b, *71*, 544.
LASKOWSKI, M., J. biol. Chem. 1942, *145*, 457.
MACINTOSH, F. C., Quart. J. exp. Physiol. 1938, *28*, 87.
MAGNUS, R., Arch. f. Physiol. 1904, 102, 123.
MARCOU, I., Kongressbericht II des XVI internat. Physiologen-Kongresses, Zürich, 1938.
MARCOU, I., E. ATHANASIU-VERGU, D. CHIRICÉANU, G. COSMA, N. GINGOLD & C.-C. PARHON, Presse med. 1938, *46*, 371.
McHENRY, E. W. & G. GAVIN, Biochem. J. 1932, *26*, 1365.
McHENRY, E. W. & G. GAVIN, Biochem. J. 1935, *29*, 622.
NEUMANN, H. & J. B. EBBINGE, Nederl. Tijdschr. Verloskde en Gyn. 1942, *45*, III, 193.
ROSE, B., S. KARADY & J. S. L. BROWNE, Amer. J. Physiol. 1940, *129*, 219.
SNEDECOR, G. W., »Statistical Methods». Ames, Iowa, 1937.
STEPHENSON, N. R., J. biol. Chem. 1943, *149*, 169.
SWEDIN, B., Nord. Med. 1943 a, *17*, 513.
SWEDIN, B., Acta Med. Scand. 1943 b, *114*, 210.
SWEDIN, B., Arkiv för kemi, mineralogi och geologi. Stockholm 1944, *17A*, 1.
TARRAS-WAHLBERG, B., Skand. Arch. Physiol. 1936, *73*, suppl. IV.
UNGAR, G. & Y. L. PARROT, C. R. Acad. Sci. Paris 1939, *208*, 598.
WERLE, E., Biochem. Z. 1940 a, *304*, 202.
WERLE, E., Biochem. Z. 1940 b, *306*, 264.
WERLE, E., Biochem. Z. 1941, *309*, 61.
WERLE, E. & G. EFFKEMANN, Arch. Gynäkol. 1940 a, *170*, 82.
WERLE, E. & G. EFFKEMANN, Klin. Wschr. 1940 b, 717.
WERLE, E. & G. EFFKEMANN, Arch. Gynäkol. 1942, *172*, 448.
WESTBERG, V., Acta obstetr. et gynecol. Scand. 1941, *21*, 180.
YEN, A. C. H. & H. C. CHANG, Proc. Soc. exp. Biol. Med. 1933, *31*, 337.
ZELLER, E. A., Helv. chim. Acta 1938 a, *21*, 880.
ZELLER, E. A., Helv. chim. Acta 1938 b, *21*, 1645.
ZELLER, E. A., Helv. chim. Acta 1940 a, *23*, 1418.
ZELLER, E. A., Helv. chim. Acta, 1940 b, *23*, 1509.
ZELLER, E. A., Helv. chim. Acta, 1941 a, *24*, 117.
ZELLER, E. A., Helv. chim. Acta, 1941 b, *24*, 539.
ZELLER, E. A., Klin. Wschr. 1941 c, I, 220.
ZELLER, E. A., Schweiz. med. Wschr. 1941 d, *71*, 544.
ZELLER, E. A., Schweiz. med. Wschr. 1941 e, *71*, II, 1349.
ZELLER, E. A. & H. BIRKHÄUSER, Schweiz. med. Wschr. 1940, *70*, 975.
ZELLER, E. A., H. BIRKHÄUSER, H. MISLIN & MARIANNE WENK, Helv. chim. Acta 1939, *22*, 1381.
ZELLER, E. A., B. SCHÄR & S. STAEHLIN, Helv. chim. Acta 1939, *22*, 837.
ZELLER, E. A., R. STERN & MARIANNE WENK, Helv. chim. Acta 1939, *23*, 3.
ÅKERBLOM, E., Acta Physiol. Scand. 1941, *2*, 378.